Evaluation of essential oils extracted from native Ethiopian home garden plants against malarial vector *Anopheles gambiae s.l.* (Diptera: Culicidae)

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ABSTRACT

Background: Mosquitoes are perilously nuisance for human welfare due to transmission of life threatening malarial parasites. *Anopheles gambiae* is one of the important mosquitoes species transmitting causal organism of malaria in several part of Ethiopia. Eco-friendly alternative strategies are currently focussing area of research in vector control program.

Objectives: To evaluate essential oils extracted from certain home garden plants against immature and adult malarial vector mosquito species *An. gamabiae.*

Materials and methods: Home garden plants such as *Lepidium sativum, Millitia ferruginea* and *Phytolacca dodecandra* seeds were collected in and around Gondar and subjected to clevanger apparatus to extract essential oil and tested against *An. gambiae.* The efficacy of essential oil was tested at different concentration against early and late instar larvae, pupae and 3-5 days old *An. gambiae* under laboratory condition by following World Health Organization (WHO) recommended protocol.

Results: The percentage mortality of early, late instar larvae and pupae of *An. gambiae* exposed to *L. sativum* essential oil was 97, 91 and 99% respectively. The early instar larva and pupa exposed at 100 ppm concentration of *M. ferruginea* oil after 72hr exposure period 100% mortality was recorded. The time recorded for knock-down effect of 50% (KT_{50}) of adults mosquitoes exposed to 10% concentration of *L. sativum* and *M. ferruginea* was 10.05 ± 0.5 and 7.34 ± 0.2 min. respectively. The female adult mosquitoes exposed to 10 ppm concentration of

L. sativum and *M. ferrugina* oil extract after 24 hr exposure period showed 82.66 and 95.66% mortality respectively.

Conclusion and recommendation: These two plant essential oil contains toxic secondary metabolites to kill immature and adult *An. gambiae* mosquitoes. However, in order to validate laboratory findings in the field appropriate formulation of essential oil is important to apply on breeding sites and indoor residual spray to control malaria vector, *An. gambiae.*

Key words/phrase: *Anopheles gambiae,* essential oil, knockdown, toxicity.

INTRODUCTION

Mosquitoes are responsible for numerous vector borne diseases associated with humans worldwide. Malaria is one of the life-threatening parasitic diseases transmitted by infected Anopheles mosquito to humans through blood feeding. The estimated annual incidence of malaria cases to be 207 million and 627,000 deaths globally, with the vast majority of cases (80%) and deaths (90%) occurring in sub-Saharan Africa (WHO, 2013). Ethiopia is the second most populous country in Africa; about 68% of the total population are living at risk of malaria (FMOH, 1999; Adhanom *et al.,* 2006). In 2005, Ethiopia embarked on a major scale-up of malaria prevention and control measures including: distribution of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS) of insecticide, use of rapid diagnostic tests (RDTs) for confirmatory diagnosis of malaria, and introduction of artemisinin-based combination therapy (ACTs). These interventions reduced the national prevalence of *Plasmodium* infection to less than 1% (FMOH, 2011). Even though, malaria transmission in Ethiopia occurs with significant spatial, seasonal, and inter-annual variation (Alemu *et al.,* 2013).

According to Killeen *et al.* (2002) mosquito control strategies targeting at immature stages in their breeding sites in sub-Saharan Africa could be highly effective, complementary to adult control interventions and should be prioritized for further development, evaluation and implementation as an integral part of rolling back malaria. Larval control of mosquitoes either by source reduction through environmental manipulation, application of larvicides in their breeding sites or a combination of both is a preferred method for reducing adult

mosquitoes in many areas of the world (Mulla *et al.*, 2001). These control strategies have some major advantage because it prevent multiplication and dispersal of adult mosquitoes thereby disease causal organism transmission reduced (Killeen *et al*, 2002).

Synthetic chemical insecticides are practiced several decades in vector control program. The indiscriminate application of chemical insecticides created countless problems such as ecological interruption, malaria vector developed resistance, bioaccumulation through food chain. In order to protect environment and control vector mosquitoes it is important to search environmentally safer, low cost technology. During the last decade, various studies conducted on natural plant products against mosquito vectors indicate their utilization as a possible alternative to synthetic chemical insecticides (Promsiri *et al.*, 2006). Plant product can be obtained either from the whole plant or from specific part by extraction with different types of solvents such as water, methanol, chloroform, hexane, etc. depending on the polarity and dissolving nature of the phytochemical (Mittal and Subbarao, 2003).

The plant extracts from variety of plants species have been demonstrated to exhibit toxicity activity against some mosquitoes. Insecticidal activity of several plant secondary metabolites such as alkaloids, steroids, terpenoids, essential oil and phenols isolated from various parts of the plants are well reviewed (Shaalan *et al.,* 2005). Mudalungu *et al.,* (2013) observed the potential larvicidal activity of both essential oil and non-volatile compounds from *Fagaropsis angolensis* leaves against *An. gambiae* larvae. Larvicidal and repellent effects of the essential oil extracted from the seeds and leaves of *Chenopodium ambrosoides* was studied against larvae and adults of *An. gambiae* mosquitoes (Bigoga *et al.,* 2013).

The immature filarial vector *Culex quinquefasciatus* exposed to solvent extract of *Phytolacca dodecandra* berries showed higher larval mortality (Nurie Misganaw *et al.,* 2012). Dose depended mortality was observed in immature and adult *Anopheles arabiensis* exposed to crude methanol extracts of *Millitia ferruginea* seed extract (Abiy Andemo *et al*., 2014). The secondary metabolite rotenone found in the seed and stem bark of *M. ferruginea* was reported to be contact and stomach poison (Salim *et al*., 2008). Therefore, biopotential plants are

considered as a source for the development of novel biopesticides to control vector mosquitoes.

STATEMENT OF THE PROBLEM

Malaria is one of the challenging problems in Ethiopia affect health of the people. The anthropogenic activities, landscape pattern and climatic conditions are highly favorable for multiplication of malarial vector mosquitoes. Seasonal rainfall in the country provides rooms for multiplication of different species of Anopheles mosquitoes. Current vector control program by using synthetic chemical pesticides reported to have several environmental and health problems in addition with resistance developed by malarial vectors. Due to this situation in order to find out eco-friendly alternative vector control strategies current study was initiated in line with global scientific research.

OBJECTIVES: The objective of this investigation was to evaluate immature and mature mosquitocidal activity of essential oils extracted from *Phytolacca dodecandra, Mellitia ferruginea,* and *Lepidium sativum.*

MATERIALS AND METHODS

Rearing of *Anopheles gambiae* mosquitoes

The larvae of *An. gambiae* were collected from suspected breeding sites from Kolla Diba region, North West of Ethiopia. In this region was known for its successive malaria epidemic with high mortality. The immature stages were collected from breeding habitat, transferred to large plastic container and transported to General Entomology laboratory, Maraki campus, University of Gondar. In the laboratory, homogenous immature stages was separated and allowed to acclimatize in deionised water. Four larval stages (I^{st}, II^{nd}, III^{rd} and IV^{th} instar) was identified based on the size of the larva and morphological features peculiar to each stage as described by Walker and Lynch (2007).

The *An. gambiae* was identified from other *Anopheles* complex species based on morphological characters by using identification key for adults and larvae. Larvae were provided with powdered dog biscuit and yeast powder (3:1 ratio) as a feed. Once the larvae reached pupal stage pupa was collected and kept in an adult emergence cage. After adult

emergence, adults was provided with *ad libitum* access of 10% sugar solution (w/v) as a feed and kept in Bugdorm cages (30 cm x 30 cm x 30 cm; Mega View Science, Taiwan). The culture was maintained at 27 ± 1°C, 65–70% relative humidity and a 12:12 h light : dark photo-period cycle. For this laboratory experiment sufficient number of larvae was collected from the field and allowed to develop and utilized.

Plant materials collection and extraction of essential oils

Three selected plants species for this study such as *Phytolacca dodecandra, Millitia ferruginea* and *Lepidum sativum* was used as traditional medicine by local people or healers. Fresh seeds were collected from each plant, washed thoroughly with distilled water and dried by spreading them on a clean and well-ventilated surface. After complete drying, plants were ground into fine powder using an electric blender. Two hundred grams (200 g) of powder from each plant sample was mixed with 1000 ml of distilled water in a conical flask. The plant parts were subjected to essential oil extraction by hydro-distillation method at 100°C using a Clevenger apparatus for 3 hours. The extracted oil was passed through cotton wool containing anhydrous sodium sulphate to remove all moisture and stored in light insensitive glass bottles at 4 °C (Mudalungu *et al.,* 2013). From the stock oil different concentrations were prepared through serial dilution method and utilized for toxicity bioassay against *An. gambiae*.

Preparation of essential oil concentrations

Stock solution of 10,000 ppm (1% ppm) concentration was prepared by adding 1 ml of pure essential oil mixed with 1 ml of acetone and make up to 100 ml in 250 ml conical flask by adding distilled water. Four concentrations viz., 100, 75, 50, 25 ppm were prepared from the stock solution and tested for larvicidal and pupicidal activity. For filter paper imperginated bioassy, 0.5, 2.5, 5.0 and 10.0% ppm contraction was prepared and utilized to check the martality rate and knockdown effect of essential oils against adult mosquitoes. In addition, control parts contain 1 ml of 100 percent acetone and the amount of distilled water varied according to parallel concentration prepared for experiment (Xue *et al*., 2001). Each experiment was replicated four times.

Toxicity Bioassay

Larvicidal activity: Larvicidal activity of essntial oils extracted from home-garden plants were evaluated by using standard World Health Organization larval susceptibility test procedure (WHO, 1996). Twenty-five early and late larval instars were released in to 250 mL plastic beaker individually. In each beaker, concentration of essential oil maintained at 0.00, 25, 50, 75 and 100 ppm concentration with the final water volume of 200 ml. These experiments were conducted at optimum temperature and relative humidity by using humidifier in mosquitoes rearing room. The larval mortality rates were recorded at 24, 48 and 72 hr exposure period (Madhu *et al.,* 2011).

Pupicidal activity: Freshly emerged pupae were used for pupicidal activity. The concentration of plant essential oils and methods were followed as mentioned in larvicidal activity. For each concentration, 25 numbers of freshly emerged pupae were released into plastic beaker and the percentages of mortality were recorded after 24, 48 and 72 hr exposure period.

Adulticidal activities: The knock-down effect of essential oil against adult mosquitoes was performed according to WHO protocol (WHO, 1996). Based on the preliminary screening results, four different concentration viz., 0.5, 2.5, 5 and 10% ppm was prepared from essential oils for experiment and control papers were treated within bendocarb (0.1%) and placed in to WHO adult test kit. Twenty five three to five days old female sugar fed adult mosquitoes were exposed on treated paper for one hour. The knock down and live mosquitoes were recorded at every 5 minute intervals. After one-hour exposure period, mosquitoes were transferred into recovery test tubes for 24 hr mortality observation. The experiments were replicated four times. The percentage of mortality recorded in larvicidal, pupicidal and adult was corrected by using Abbott's formula (Abbott, 1925). Corrected % mortality = (% mortality in test - % mortality in control)/ (100-% mortality in control) X (100).

Statistical analysis: The percentage corrected mortality of *An. gambiae was* obtained from four replications at four different concentrations and control was subjected to statistical analysis. The calculations were carried out by using Microsoft Excel program in order to

obtain mean percentage values and standard error. The SPSS version 20 software was used to confirm statistical significant at 5% level ($P < 0.05$) by using *Chi*-square ($\chi 2$) analysis. The LC_{50} and LC_{90} values and 95% upper confidence limit (UCL) and lower confidence limit (LCL) were also calculated.

RESULTS

Toxicity of *Lepidium sativum* essential oil against *An. gambiae*

Mean percentage of mortality of larvae and pupae of *An. gambiae* mosquitoes exposed to *L. sativum* plant essential oil after 24, 48 and 72 hr exposure period was presented in Table 1. In early instar larvae, maximum percentage mortality of 96.00% was observed at 100 ppm after 24 hr exposure period. The calculated LC_{50} and LC_{90} values was 317.8 and 437.1 ppm respectively. The $\chi 2$ analysis results showed stastical significance at 5% level ($\chi 2 = 122.57$; $p = 0.000$). The calculated range of 95% LCL and UCL of LC_{50} and LC_{90} value was 297.8 – 337.0 and 404.5 – 491.0 ppm respectively. In late instar larvae, maximaum percentage mortality of 98.00% was observed at 100 ppm after 72 hr exposure period. The calculated LC_{50} and LC_{90} value was 337.3 and 466.4 ppm respectively. The $\chi 2$ analysis results showed stastical significance at 5% level ($\chi 2 = 1371.59$; $p = 0.000$). The calculated range of 95% LCL and UCL of LC_{50} and LC_{90} value was 2101.1 - 456.5 and 379.0 – 2830.4 ppm respectively. In pupae, maximaum percentage of mortality of 99.00% was observed at 100ppm after 72 hr exposure period. The calculated LC_{50} and LC_{90} value was 245.3 and 344.2 ppm respectively. The $\chi 2$ analysis results showed stastical significance at 5% level ($\chi 2 = 792.39$; $p = 0.000$). The calculated range of 95% LCL and UCL of LC_{50} and LC_{90} value was 186.8 – 297.3 and 286.5 – 600.1 ppm respectively

Table 1: Percent mmortality of immature *An. gambiae* exposed *Lepidium sativum* after 24, 48 and 72 hr exposure period.

An.gambiae stages	Dose (ppm)	Exposure time		
		24 hr	48 hr	72 hr
Early instars	Control	0.00 ± 0.00	1.00 ± 1.00	5.00 ± 1.91
	25 ppm	10.00 ± 2.58	19.00 ± 2.88	83.75 ± 2.01
	50 ppm	26.00 ± 2.16	58.50 ± 3.30	94.00 ± 2.58
	75 ppm	91.00 ± 2.00	96.00 ± 3.26	96.00 ± 1.63
	100 ppm	96.00 ± 1.62	97.00 ± 3.82	97.00 ± 1.91

	LC_{50} (LCL - UCL)	317.8 (297.8 - 337.0)	267.7 (253.7 – 281.3)	160.4 (136.6 – 182.7)
	LC_{90} (LCL - UCL)	437.1 (404.5 – 491.0)	389.9 (365.9 – 423.9)	261.7 (226.6 – 325.1)
	$\chi 2$	122.57	62.43	204.94
	P value	P = 0.000	P = 0.000	P = 0.000
Late instars	Control	0.000 ± 0.00	1.00 ± 1.00	2.00 ± 1.15
	25 ppm	5.00 ± 1.91	7.00 ± 1.91	8.00 ±1.63
	50 ppm	20.00 ± 4.32	29.5.50 ± 6.50	11.00 ± 1.91
	75 ppm	47.00 ± 5.18	60.00 ± 1.63	86.50 ± 5.50
	100 ppm	74.00 ± 2.58	88.00 ± 1.63	91.00 ± 3.26
	LC_{50} (LCL - UCL)	403.3 (385.5 - 423.5)	354.2 (331.0 - 378.7)	337.3 (210.1 – 456.5)
	LC_{90} (LCL – UCL)	653.7 (597.1 - 740.5)	564.0 (507.0 - 684.0)	466.4(379.0– 2830.4)
	$\chi 2$	44.93	100.19	1371.59
	P value	P = 0.000	P = 0.000	P = 0.000
Pupae	Control	0.00 ± 0.00	2.00 ± 1.91	6.00 ± 2.58
	25 ppm	3.00 ± 1.91	6.00 ± 2.58	7.00 ± 1.91
	50 ppm	33.50 ± 2.98	46.50 ± 3.86	85.00 ± 1.91
	75 ppm	91.00 ± 2.51	96.00 ± 1.63	98.00 ± 1.15
	100 ppm	93.00 ± 4.43	94.00 ± 3.82	99.00 ± 1.00
	LC_{50} (LCL – UCL)	321.5 (301.7 - 340.7)	292.9 (241.0 – 340.2)	245.3 (186.8 – 297.3)
	LC_{90} (LCL – UCL)	429.1 (398.0 – 481.5)	224.0 (360.6 – 635.1)	344.2 (286.5 – 600.1)
	$\chi 2$	133.53	566.53	792.39
	P value	P = 0.000	P = 0.000	P = 0.000

Toxicity of *Millittia ferruginea* essential oil against *An. gambiae*

Mean percentage mortality of larvae and pupae of *An.gambiae* mosquitoes exposed to *M. ferruginea* plant essential oil after 24, 48 and 72 hr exposure period was presented in Table 2. In early instar larvae, maximaum percentage of mortality of 100.00% was observed at 100 ppm after 48 hr period. The calculated LC_{50} and LC_{90} value was 285.6 and 353.0 ppm respectively. The $\chi 2$ analysis results showed stastical significance at 5% level ($\chi 2 = 36.75$; p = 0.000). The calculated range of 95% LCL and UCL of LC_{50} and LC_{90} value was 277.1 – 293.5 and 340.9 – 369.1 ppm respectively.

In late instar larvae, maximaum percentage mortality of 96.00% was observed at 100 ppm after 72 hr exposure period. The calculated LC_{50} and LC_{90} value was 284.2 and 457.7 ppm respectively. The $\chi 2$ analysis results showed stastical significance at 5% level ($\chi 2 = 80.09$; p = 0.000). The calculated range of 95% LCL and UCL of LC_{50} and LC_{90} value was 265.6 – 302.4 and 419.2 – 516.3 ppm respectively. In pupae, maximaum percentage of mortality of 100.00% was observed at 100 ppm after 72 hr exposure period. The calculated LC_{50} and LC_{90} values was not calculated by SPSS software due to no variation in percentage of mortality. The $\chi 2$ analysis results showed stastical significance at 5% level ($\chi 2 = 3178.7$; p = 0.000). The range of 95% LCL and UCL of LC_{50} and LC_{90} value was not calculated.

Table 2: Percent mortality of immature *An. gambiae* exposed *Millitia ferruginea* after 24, 48 and 72 hr exposure period.

An.gambiae stages	Dose (ppm)	Exposure time		
		24hr	48hr	72hr
Early instars	Control	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 1.91
	25 ppm	2.00 ± 1.15	4.00 ± 1.63	7.00 ± 1.91
	50 ppm	26.00 ± 2.16	58.50 ± 6.60	94.00 ± 2.58
	75 ppm	91.00 ± 2.00	99.00 ± 2.00	100.00 ± 0.00
	100 ppm	98.00 ± 2.30	100.00 ± 0.00	100.00 ± 0.00
	LC_{50} (LCL - UCL)	327.5(317.1- 337.9)	285.6 (277.1 - 293.5)	Not calculated
	LC_{90} (LCL - UCL)	408.2(391.1- 431.8)	353.0 (340.9 -369.1)	Not calculated
	$\chi 2$	52.78	36.75	2183.21
	P value	P = 0.000	P = 0.000	P = 0.000
Late instars	Control	0.000 ± 0.00	1.00 ± 1.00	2.00 ± 1.15
	25 ppm	5.00 ± 1.91	18.00 ± 2.58	19.50 ±2.62
	50 ppm	23.00 ± 3.41	34.50 ± 2.98	45.50 ± 3.30
	75 ppm	47.50 ± 5.18	60.00 ± 3.26	86.50 ± 2.75
	100 ppm	79.00 ± 2.58	88.00 ± 1.63	96.00 ± 1.63
	LC_{50} (LCL - UCL)	400.0(383.3- 418.7)	334.7 (315.3 - 355.4)	284.2(265.6- 302.4)
	LC_{90} (LCL – UCL)	659.6(604.9- 740.6)	609.8 (548.8 - 704.4)	457.7(419.2- 516.3)
	$\chi 2$	37.9	56.47	80.09
	P value	P = 0.000	P = 0.000	P = 0.000
Pupae	Control	0.00 ± 0.00	3.00 ± 1.91	6.00 ± 2.58
	25 ppm	3.00 ± 1.91	6.00 ± 2.58	7.00 ± 1.91
	50 ppm	8.00 ± 1.63	11.00 ± 1.91	15.00 ± 1.91
	75 ppm	91.00 ± 2.51	96.00 ± 1.63	98.00 ± 1.15
	100 ppm	97.00 ± 3.82	99.00 ± 1.91	100.00 ± 0.00
	LC_{50} (LCL – UCL)	351.8(322.0- 380.9)	Not calculated	Not calculated
	LC_{90} (LCL – UCL)	446.6(407.6- 534.0)	Not calculated	Not calculated
	$\chi 2$	298.87	6388.67	3178.78
	P value	P = 0.000	P = 0.000	P = 0.000

Toxicity of *Phytolacca dodecandra* essential oil against *An. gambiae*

Mean percentage of mortality of larvae and pupae of *An. gambiae* mosquitoes exposed to *P. dodecandra* plant essential oil after 24, 48 and 72 hr exposure period was present in Table 3. In early instar larvae, maximaum percentage of mortality of 10.00% was observed at 25 ppm after 24 hr exposure period. The calculated LC_{50} and LC_{90} values was not calculated by SPSS software due to no variation in percentage of mortality. The $\chi 2$ analysis results did not showed stastical significance at 5% level ($\chi 2 = 41.09$; $p = 0.214$). In general percentage mortality of different stages of *An. gambiae* exposed to *P. dodecandra* essential oil at different concentration was minimum and the result was statistically not significant at 5% level ($P > 0.05$).

Table 3: Percent mortality of immature *An. gambiae* exposed *Phytolacca dodecandra* after 24, 48 and 72 hr exposure period.

An.gambiae stages	Dose (ppm)	Exposure time		
		24hr	48hr	72hr
Early instars	Control	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 1.91
	25 ppm	10.00 ± 5.16	10.00 ± 2.88	10.50 ± 2.21
	50 ppm	6.00 ± 5.16	8.00 ± 2.32	8.50 ± 1.7
	75 ppm	4.00 ± 3.26	6.00 ± 1.15	9.00 ± 1.00
	100 ppm	2.50 ± 1.62	5.00 ± 2.08	9.00 ± 1.00
	LC_{50} (LCL - UCL)	Not Calculated	Not Calculated	Not Calculated
	LC_{90} (LCL - UCL)	Not Calculated	Not Calculated	Not Calculated
	$\chi 2$	41.09	81.18	33.15
	P value	P = 0.214	P = 0.118	P = 319
Late instars	Control	0.000 ± 0.00	1.00 ± 1.00	2.00 ± 1.54
	25 ppm	5.00 ± 1.91	6.00 ± 1.75	7.00 ±1.00
	50 ppm	2.00 ± 1.15	4.00 ± 1.63	4.00 ± 1.63
	75 ppm	4.50 ± 2.06	5.00 ± 2.0	7.00 ± 1.29
	100 ppm	5.00 ± 1.73	6.50 ± 1.89	5.00 ± 1.91
	LC_{50} (LCL - UCL)	Not Calculated	Not Calculated	Not Calculated
	LC_{90} (LCL – UCL)	Not Calculated	Not Calculated	Not Calculated
	$\chi 2$	64.769	45.105	30.879
	P value	P = 0.143	P = 0.174	P = 0.053
Pupae	Control	0.00 ± 0.00	0.00 ± 0.00	6.00 ± 2.58
	25 ppm	3.00 ± 1.91	4.00 ± 1.63	5.50 ± 0.95
	50 ppm	5.50 ± 2.75	6.50 ± 2.50	6.50 ± 2.50
	75 ppm	5.00 ± 2.51	2.00 ± 1.63	7.00 ± 1.00
	100 ppm	5.00 ± 1.91	6.50 ± 1.50	7.50 ± 1.25
	LC_{50} (LCL – UCL)	Not Calculated	Not Calculated	Not Calculated
	LC_{90} (LCL – UCL)	Not Calculated	Not Calculated	Not Calculatd
	$\chi 2$	88.73	68.11	34.32
	P value	P = 0.336	P = 0.389	P = 0.940

Knock down time (KT_{50}) for adult *An. gambiae* exposed to plant essential oils

Three essential oils were identified as potential treatments for knock-down activity in filter paper impregnated WHO adult test kit and the results were presented in Table 4. After 1 hr exposure period at 10% of *Milletia ferruginea* essential oil recorded 7.34 ± 0.25 min. which was the lowest KT_{50} value among the three essential oil tested. The time taken for 50% adult mosquitoes knock down at 10% concentration of *Lepidium sativum* was 10.05 ± 0.5 min. The KT_{50} value recorded in control (bendocarb 0.1%) was 22.75 min. Among the three essential oils tested minimum time taken for knock down effect was recorded in *Milletia ferrruginea* at 2.5, 5 and 10% compared to control.

Table 4: Knock down time (KT_{50}) recorded for *An. gambiae* exposed to plant essential oils.

Essential oil	KT_{50} (Mean ± SE) min

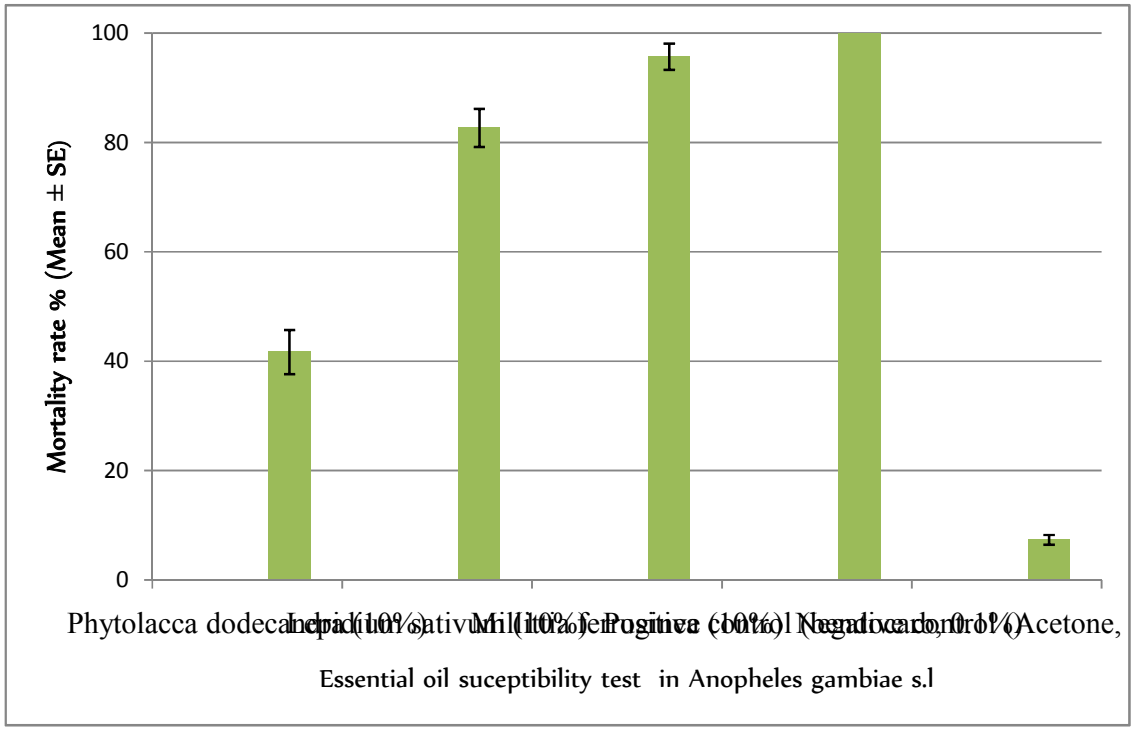
tested	0.5%	2.5%	5%	10%
Lepidium sativum	31.13 ± 0.6	23.05 ± 0.2	13.25 ± 0.1	10.05 ± 0.5
Millettia ferruginea	35.15 ± 0.4	19.7 ± 0.6	11.15 ± 0.2	7.34 ± 0.2
Phytolacca dodecandra	55.05 ± 0.2	52.01 ± 0.1	46.15 ± 0.6	38.25 ± 0.2
Conrol (Bendocarb 0.1%)			22.75	

Values are mean ± standard error of four replication. KT_{50} = Knock down times required to kill 50% of the population exposed for 1 hr.

Mortality of adult *An. gambiae* exposed to plant essential oil

The percentage mortality of *An gambiae* exposed to essential oil impregnated paper by using WHO adult test kit was presented in Figure 1. Results revealed that maximum percentage mortality of 95.6% was recorded from *Millettia ferruginea* essential oil. The percentage mortality was 82.6% in *Lepidium sativum* essential oil compared to negative control acetone.

Figure 1: Percentage mortality of adults *An. gambiae* exposed to plant essential oils



DISCUSSION

Mosquitoes are transmitting various diseases from organism to human and animal. Mosquito management at their life cycle is important to minimize the emergence of adult population thereby reduce the vector-borne disease incidence. Mosquito control in the larval stages in small breeding habitats is easy control. Those mosquitoes breeding sites includes small ponds, marshes, ditches, pools, drains, water containers and any other utensils holding water is easily manageable. Eco-friendly alternatives are important for safer control of mosquitoes. The essential oil from plant origin was proved to be effective due to multiple modes of action (Ghosh *et al*., 2008). The present results confirmed high toxic effect of essential olis extracted from *L. sativum, M. ferrugina* against immature and adults *An.gambiae* mosquitoes. The percentage *larval* and pupal and adult mortality were varied significantly based on the plant species, concentration and exposure period.

Among the three essential oils tested, mortality rate of early, late instar larvae and pupal stage was recorded maximum from *L. sativum* and *M. ferruginea.*. The variation of mortality was associated with toxic secondary metabolites accumulated in plant species. Toxic chemical substances found in medicinal plants completely varied from species to species, geographical distribution and also extraction methdology. The percentage mortality in all stages of immature mosquitoes was increased in increased concentration and period of exposure. This highlights that selection of plant species and appropriate concentration and exposure period was important for maximum benefits of plant extract in mosquito control program.

Essential oil of *M. ferruginea* showed 100, 96 and 100% mortality in early, late larvae and pupae after 72 hrs esposer at 100 ppm concentration. Similar results was reported earlier by Abiy Andemo *et al*. (2014) by using methanol extract of *M. ferruginea* seeds against third and fourth instar larvae and adults of *An. arabiensis.* Tomass *et al*. (2011) also reported using methanol extracts of *Jatropha curcas* against *An. arabiensis* larvae under laboratory condition. Essential oil of *L. sativum* showed second highest mortality rate of 97, 91 and 99% in early, late larvae and pupae after 72hrs exposure period at 100 ppm concentration. In addition, results of the present study revealed that larvae, pupae and adult mosquito mortality increased with increasing concentration at the same exposure time This result was supported by (Kimbaris, *et al*., 2012) they have observed dose depended mortality from plant bioactive

compounds against the West Nile vector *Culex pipiens*. The toxic effect of *P. dodecandra* essential was minimum against *An. gambiae.*

This results conclude, that essential oils extracted from the seeds of *L. sativum* and *M. ferruginea* showed promising mosquitocidal activity against all stages of the primary malaria vector *An. gambiae.* Recent time's *An. gambiae* was developing resistance to most insecticides used in malaria vector control program in Ethiopia. These botanicals could be a useful alternative in malaria vector control program. In addition, botanicals can be used for targeted larval control in selected temporary breeding habitats to reduce toxic effect on non-target organisms and environmental contamination. The bioactive compounds are by products of steam distillation of essential oils can be easily obtained from distillation process. However, appropriate formulation need to be developed for large scale application and also to confirm the bio-potential of these essential oil with in-depth field trial after formulation.

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Diversity and Symbiotic Effectiveness of *Rhizobium* Isolates Collected from different Faba bean (*Vicia faba*) growing areas of North and South Gondar, Ethiopia

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Abstract

Biological nitrogen fixation is a natural process for fixation of molecular nitrogen to maintain sustainable agriculture throughout the world. Thus, the aim of this study was to isolate, characterize and select potential *Rhizobium* strains collected from different faba bean growing areas of North and South Gondar, Ethiopia. About 3kg soil samples from each site with previous history of faba bean cultivation was collected and brought to the laboratory for further analysis. A total of 57 *Rhizobium* isolates were isolated based on infection method. The isolates were characterized on Yeast Extract Mannitol Agar (YEMA) medium and authentication of strain test was done on sand culture using pot experiment. The isolates attain colony sizes ranging from 1.5 to 4.5 mm after 3 to 5 days of incubation. All the isolates showed growth on all the tested carbon sources except lactose, arabinose and mannose. Most of the isolates showed growth at different pH levels ranging 5 to 9, salt concentrations from 0.1% to 2%, temperatures between 5 and 45°C and at different antibiotics with different concentrations. In sand culture, only 35.5% of isolates showed nodulation. Analyses of variance indicated, inoculation of isolates improves tested traits significantly ($p< 0.05$) at all measured investigated parameters such as shoot length, shoot dry weight, and plant total nitrogen as 11%, 28% and 31.3%, respectively, over nitrogen treated plants, 2%, 10% and 29.4% respectively over standard *Rhizobium* and 55%, 82.3% and 85.7% respectively over negative treatments. Shoot dry weight was found to be strongly positively correlated with symbiotic effectiveness ($r= 0.994$, $P <0.01$). Based on symbiotic effectiveness, 80% of the tested isolates was found to be highly effective, 13% effective and only one isolate less effective. From this finding, most of our isolates displayed abundant diversity in their response to morphological and physiological characteristics. Inoculation of selected *Rhizobium* isolates revealed shoot dry weight enhancement over nitrogen treated plants of faba bean on sand culture using pot experiment under controlled greenhouse condition. Dendrogram analysis shows that isolates were categorized into *six* major clusters that were again sub clustered to many. Best isolates for various agro-climatic regions were identified.

Therefore, there is a need for detail study of effective isolates tested on field condition and molecular characterization for better classification of the *Rhizobium* isolates.

Key words: Nitrogen fixation, Legumes, *Rhizobium*, Total nitrogen, Authentication

INTRODUCTION

Ethiopia is a home to about a dozen species of tropical grain legumes. An estimated 1.5 million ha of land is planted to grain legumes in this country and more than 1.9 million metric tons (MT) of grain produced each year, touching the lives of about 10 million households (Ronner *et al*., 2012). Grain legumes have a nitrogen fixing symbiosis with soil root nodule bacteria. Among these, Faba bean (*Vicia faba* L.) is an annual grain legume widely cultivated, which serves as foods for human and animal nutrition in many countries, since it is rich in protein, minerals and vitamins. Cultivation of faba bean plays important roles in maintaining sustainable agriculture system in many marginal areas; due to its high nutritional value, multiple uses and ability to grow over a wide range of climatic and soil conditions.

Faba bean, considered to be one of the nine founder crops (or primary domesticates), is probably native to southwestern Asia. After its domestication at least 7000 years ago, faba bean spread to Africa, the Mediterranean basin and central Europe, the Americas, China and India. China and Europe are the main producers, contributing 43.0% and 20%, respectively, of the world faba bean production (FAOSTAT, 2008).

Ethiopia is one of the largest faba bean producing countries in the world only second to China. Historically, Ethiopia is considered as the secondary center of diversity and also one of the nine major agro-geographical production regions of faba bean. Faba bean (*Vicial faba L.*) is the leading grain legume crop grown as field crop, a daily food and cash crop throughout the highlands between the altitudes 1800 and 3000 masl in Ethiopia (Temesgen and Aemiro, 2012).

Legumes are able to establish nitrogen-fixing symbioses with bacterial microsymbionts (rhizobia), thus reducing the need for chemical fertilizers. This reduction may help to minimize greenhouse- gas emissions and to avoid contamination of ecosystems. This association further provides a nitrogen supplement for the subsequent crops. Faba bean (*Vicia*

faba), field pea (*Pisum sativum*) and lentil (*Lens culinaris*) together with chick pea (*Cicer arietinum*) are the major highland pulse crops in Ethiopia and occupy 12 to 15% of the land under cultivation (ICARDA, 2012). These leguminous crops are known for their ability to form endosymbiotic relationship with the root nodule bacteria collectively called Rhizobia. Faba bean like other legumes crops has the ability to form symbiotic association with root nodulating bacteria (rhizobia) group called *Rhizobium leguminosarum bv. viciae.* Faba bean mainly grown for its high protein content which accounts about 30 % on average and it is vital nutrient for human and animals as source of protein (Kamal *et al*., 2012). Faba bean popularity has increased recently as its high yield makes it attractive to producers while its high protein content and low-priced makes it attractive to consumers.

In Ethiopia, for the last three decades attempts have been made to conduct research on cool season legumes. Identification using a few phenotypic characters were undertaken to identify several isolates of *Rhizobium leguminosarum bv. vicieae* (Zerihun and Fassil, 2011) from few regions of Ethiopia. However, reports on rhizobial polyphasic characterization approach and selection of potential *Rhizobium leguminosarum bv. viciae* that show superior in nitrogen fixing capacity are still insufficient. Hence, the present study aims to identify efficient *Rhizobium* isolates and their effect on nodulation isolated from the major faba bean producing areas of different districts of North and South Gondar using polyphasic characterization approach.

OBJECTIVES

General Objective:

- ✓ To examine the diversity and symbiotic effectiveness of *Rhizobium leguminosarum bv. viciae* isolates collected from different Faba bean (*Vicia faba*) growing areas of North and South Gondar zones.

Specific objectives: The specific objectives of this study are to:

- ✓ Isolate *Rhizobium* strains collected from different locations of North and South Gondar.
- ✓ Characterize different isolates of *Rhizobium* using morphological features, biochemical tests and physiological tests.

- ✓ Select the potential *Rhizobium* strains for farmers that show superior in their nitrogen fixation capacity.

MATERIAL AND METHODS

The presumptive test, *Rhizobium* isolation, identifications and pot experiments were carried out at the Department of Biotechnology, University of Gondar. Plant total nitrogen and Soil chemical analysis were done at Adet Agriculture Research Centre.

Collection and Identification of Soil sampling

Three representative faba bean growing woredas were selected from North Gondar and South Gondar zone (Lay Gayint and Farta woreda from South Gondar; Chilga from North Gondar) based on their productivity of faba bean in the last few years. From each woreda, the productive areas were selected based on their productivity of faba bean. From each woreda of productive area, representative farmer fields were selected based on the previous history of cultivating faba bean crop. Thus, twenty farmer fields from Chilga, a twenty farmer fields from Farta and seventeen farmer fields from Lay Gayint were selected as shown on (Fig. 1). About 3 Kgs of soil sample was collected from each selected farmer fields at a depth of 20 cm using sterile (fresh) plastic bags.

Colony Morphological and Biochemical Characterization: Colony morphology was characterized on YEMA according to Jordan (1984). Gram staining and acid/base reactions were evaluated on YEMA containing 25 μg ml^{-1} bromothymol blue (BTB) (Lupwayi and Haque, 1994).

Utilization of Carbon source: Carbon source utilization of isolates was determined following the method of Somasegaran and Hoben (1994) on fifteen carbohydrates prepared as 10% (w/v) in sterile distilled water. Each 10 ml of the carbohydrate stock solutions were added to 90 ml of the carbohydrate free basal medium and their growth were observed after 3-5 days.

Acidity, alkalinity, salinity and temperature tolerance

All experiments on tolerance to acidity, alkalinity, temperature and salinity were performed according to McVicar *et al*. (2005). Tolerance to acidity and alkalinity of each isolate was evaluated on YEMA with at a pH adjusted to 4.0 to 9.0 with sterile 1N HCl or NaOH. For

salt tolerance, the isolates were transferred to YEMA plates supplemented with NaCl at concentrations of 0.1, 0.5, 1, 2, 3, 4, 5% (w/v). The ability of bacterial strains to grow at high and low temperatures was monitored at incubation temperatures of 5, 10, 15, 35, 40 and 45°C.

Intrinsic antibiotic resistance: The intrinsic resistance of isolates was determined by inoculating 10 µl of each culture (10^9 cells/ml)) on solid YEMA medium containing four filter sterilized with Millipore filters antibiotics at different concentrations of water and ethanol according to Taye (2010).

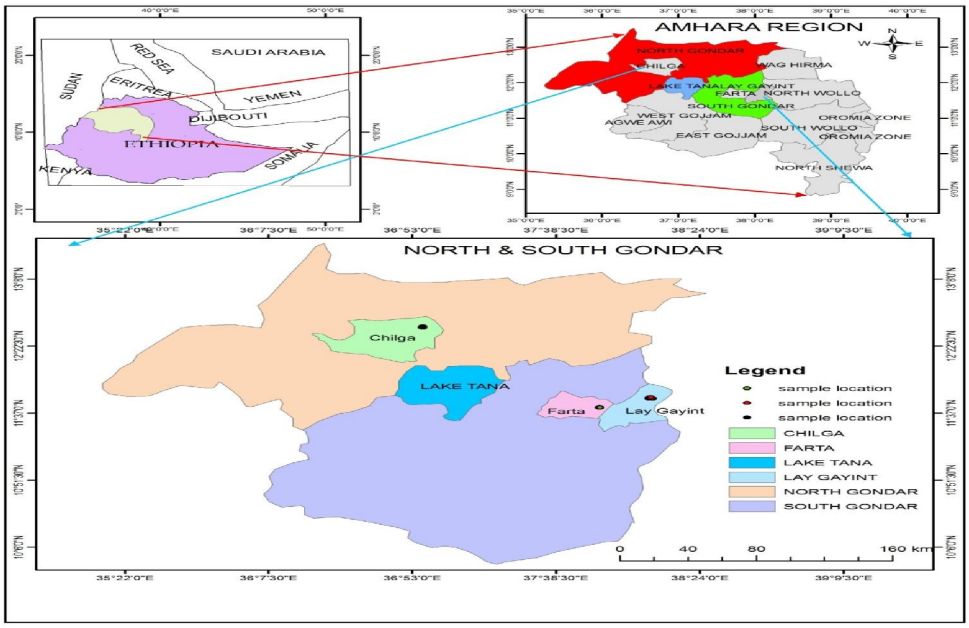


Fig.1. Map of the Soil sampling location in North and South Gondar Zones in Amhara Region

Authentication of the strains (Symbiotic effectiveness)

In order to determine the isolates performance (effectiveness) in nitrogen fixation, authentication test was done as described by Vincent (1970). Out of 57 isolates, 40 best isolates were selected from the presumptive test based on nodule color, nodule number, nodule fresh weight and shoot height. For each isolate three surface sterilized pots were filled with approximately 3kg acid washed and heat sterilized river sand. In each pot four to five healthy surface sterilized Adet faba bean variety seeds from Adet Agricultural Research Centre were germinated and planted according to the methods of Somasegarian and Hoben (1994). A total of 120 pots were required for this experiment. As a starter, 20 ppm of nitrogen

was included in each pot before planting. Each seedling was inoculated with 1 ml of each isolate with an inoculum size of 10^9 cells/ml. After a week, the seedlings were reduced into three per pot. Two treatments were used as control: one without nitrogen supply and an uninoculated (i.e. negative control) and the other an uninoculated but with provision of 0.05% (W/V) KNO_3 per week (i.e. positive control).

This experiment was made in triplicates and the plants were grown under glasshouse condition. The pots were arranged in Completely Randomized Design (CRD) and plants were also fertilized with the quarter strength Broughton and Dilworth nitrogen-free nutrient solution once a week and received water every three days. After 45 days of planting, the plants were carefully uprooted and nodule color, nodule number, nodule fresh weight, shoot length were counted and measured, nodule dry weight, root biomass and shoot dry weight were also measured after drying at 70 ºC for 24 hr under dry oven, and total nitrogen were analyzed by kjeldhal method. The Relative symbiotic effectiveness of the isolates were calculated according to the equation proposed by Purchino *et al.* (2000) (100 X inoculated plant DM/ N-fertilized plant DM) with Nitrogen fixing effectiveness classified as ineffective <35%; lowly-effective, 35 to 50%; effective, 50 to 80%; and highly effective, >80%.

Nodulation status survey: The general survey was conducted during the identification and collection of soil samples. In most cases, attempts were made to meet the owner of the field to establish cropping history and the owner of the field told or informed as, their field has been covered with faba bean crop.

Data analysis: Statistical data analysis was done by using SAS software version 9.2. Analysis of variance (ANOVA) was done for the comparison of means for all treatments and Duncan's multiple range test were used to detect the significant difference among treatment means at $p \leq 0.05$. Correlation coefficients were calculated to study the association among the measurement traits using Pearson correlations. Data from all physiological studies were also used for cluster analysis and similarities between isolates and a dendrogram was constructed based on average linkage hierarchical cluster analysis between groups using SPSS version 20.0 software statistical program**.**

RESULT AND DISCUSSION

Morphological and Biochemical characteristics

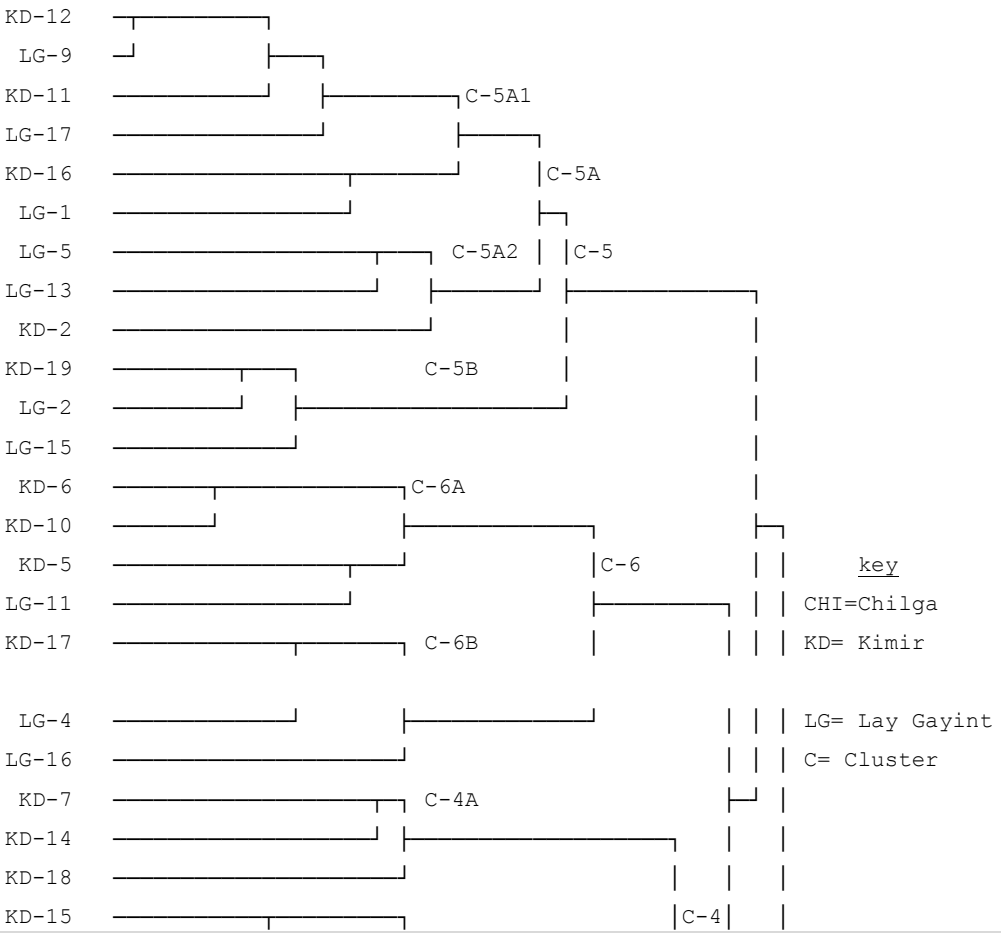
All isolates fulfilled the characteristics of rhizobia (Lupwayi and Haque, 1994): as gram-negative, rod-shaped, the color of colonies was milky-white opaque with a circular shape, regular borders and raised, showing intermediate to high production of mucus after 3 to 5 day of growth on YEMA medium at 30^0C. Furthermore, the colony diameter of all isolates of *Rhizobium leguminosarum* bv. *viciae* were within the range of 1.5-4.5mm after 3 to 5 days of incubation at 30^oc (data not shown). According to the previous report of Jordan, (1984) Rhizobium *leguminosarum* isolates usually show colony size between 2-4mm in diameter and 95 % of our isolates fall to this group (data not shown).

All the isolates changed the YEMA-BTB medium to yellow color and did not absorb Congo red on YEMA-CR media, indicate that all the isolates are acid producer and fast growing rhizobia. Similar classification has been done by Jordan, (1984). These color formation is due to the utilization of the sugar component of the medium for their growth. This finding is similar with the previous work of Aynabeba *et al.* (2001) indicating that many *Rhizobium* strains isolated from each sampling field in Semen Shewa were fast growing and acid producing. All the strains were gram negative and rod shaped as revealed by Gram's staining technique. These results confirmed the finding obtained by Tagelsir and Mohamed (2015).

Hierarchal cluster analysis: Hierarchal cluster analysis for fifty seven isolates of *Rhizobium* based on their physiological characteristics including carbon source utilization, pH tolerance, salinity tolerance, temperature tolerance and different antibiotic tolerance at different concentrations (data not shown) were used to construct a dendrogram using average linkage clustering method between groups. Based on the above parameters, *Rhizobium* isolates from different North and South Gondar locations essentially grouped in to six main clusters and the individual main cluster contains sub clusters to analyze their similarity using Pearson's coefficient on the dendrogram (Fig. 2).

The highest similarity were computed between isolate (KD-4) from Kimir Dingay and (LG-3) from Lay Gayint in cluster 4 and isolate (KD-12) from Kimir Dingay and (LG-9) from Lay

Gayint in cluster 5 which were nearly100% similar. This 100% similarity indicates, the isolates were isolated from the same soil pH, temperature, salinity, resistance of different antibiotics at different concentrations and carbon source utilization (data not shown). On the other hand, CHI-6 and CHI-19 isolates from Chilga in cluster 2, CHI-14 and CHI-20 isolates from Chilga in cluster 1 and KD-6 and KD-10 isolates from Kimir Dingay in cluster 6 were revealed 88% similarity on the dendogram. Whereas, the lowest similarity was computed between isolate CHI-15 from Chilga and isolate KD-6 from Kimir Dingay which presented in to two different clusters (i.e. CHI-15 in cluster 1 and KD-6 in cluster 6). This lowest similarity shows, the isolates were isolated from different soil pH (CHI-15 ranging 5-9 and KD-6 between 5.5-8), temperature (CHI-15 between 15-40^{0}c and KD-6 ranging from 5-35^{0}c), salt concentration (CHI-15 at 0.5 % w/v and KD-6 at 1% w/v), carbon utilization (CHI-15 uses 87% and KD-6 uses 100% of tested carbon sources) and different antibiotics at different concentrations (data not shown). Similarity increases as we read from right to left on the denogram, based on Pearson's correlation coefficient value (Fig. 2).



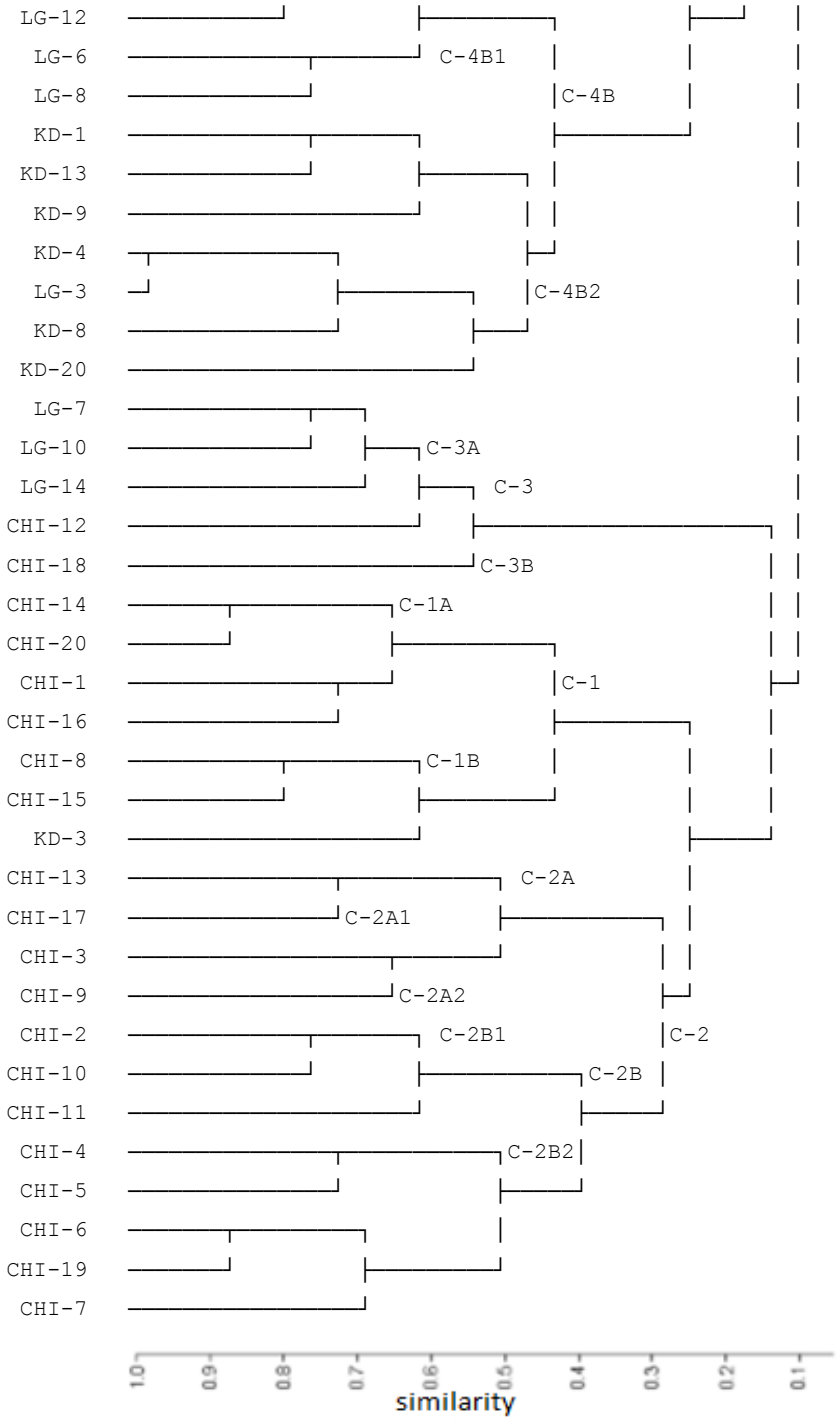


Fig. 2. Dendrogram showing the similarity using Pearson's coefficient.

Analysis of variance: Forty isolates obtained from presumptive test of faba bean nodules were assessed for their infectiveness and effectiveness of nitrogen fixation on Adet faba bean on sterile and acid treated sand in a pot experiment under Greenhouse. Out of these, only fifteen isolates formed nodules on the test faba bean root authenticating as *Rhizobium* (Table

1). The ability to form nodules along with the subsequent nitrogen fixing capacity is widely used as means of assessing the association between rhizobia and respective hosts (Brockwell *et al*., 1995).

The results of analyses of variance showed that *Rhizobium* inoculation significantly ($P<0.05$) increased at all investigated parameters such as, number of nodules per plant, nodule fresh weight, nodule dry mass, shoot dry weight, shoot length, root biomass, plant total nitrogen and symbiotic effectiveness as compared to the standard and control treatments (Table 1). This finding is similar with Dereje *et al*. (2015) symbiotic effectiveness of faba bean nodulating on sand culture and Anteneh (2012) symbiotic effectiveness of *Rhizobium leguminosarum var. viciea* nodulating lentil in Ethiopian.

The nodulating isolates showed difference with nodule number of 61.33 p^{-1} for (KD-13) and 103 for (LG-1) p^{-1} (Table 1) which were less than 67 and 168 nodule number p^{-1} of faba bean on sand culture reported by Zerihun and Fassil (2011), but higher than 17 p^{-1} for isolate AUFR-11 (Semema) to 91 p^{-1} for isolate AUFR-5 (Koyetsa) of degaga variety of faba bean on sand culture reported by Solomon and Fassil (2014). The mean nodule number p^{-1} recorded in this study was 71.4 and which was less than both 88 nodule number p^{-1} for degage and 92 nodule number p^{-1} for dosha faba beans on sand culture studied by Dereje *et al.* (2015) and 98 nodule number p^{-1} of faba bean on acidic soil reported by Girmaye *et al*. (2014). Fifty three percent of isolates showed higher number of nodules p^{-1} as compared to standard *Rhizobium* isolate from national soil laboratory, Addis Abeba.

The highest plant shoot length 48.67cm was recorded for both isolate (KD-4) and (LG-1) inoculated with Adet faba bean variety (Table 1). These improvements in shoot length were equivalent to 54.8% over the negative control (without inoculation and nitrogen supplement), 11% over the positive control (nitrogen treated plants) and over the standard *Rhizobium* 2.1% with Adet faba bean. This result was the best compared with the results of Dereje *et al*. (2015) study on faba bean inoculation with Degaga variety which was measured shoot height of 43.3 cm of isolate HUGAVf1 collected from acidic soils of Ethiopia, which was 32.94% over the negative control and 16.9% over the positive controls. Our result was also better than

the result of Anteneh (2012) study on faba bean inoculation with Degaga variety which was measured 49.7 cm shoot length of isolate NSFBR-48 collected from Central Ethiopia, which had shown noticeable improvement in shoot height of 51cm over negative control and 14% nitrogen treated plants. This improvement of shoot length could be regard as; the rhizobia may increase plant growth by providing products through nitrogen fixation (Kumar *et al*., 2014).

Plant total nitrogen ranges from 2.76 % for isolate (LG-1) to 4.29 % for isolate (KD-4) and the mean plant total nitrogen of isolates were 3.2241% (Table 1). In general, inoculation of *Rhizobium* isolates resulted in a significant difference at ($p<0.05$) in plant total nitrogen over negative treatments. Analysis of variance shows, inoculation increased the plant total nitrogen at 31.3%, 29.4% and 85.7% over nitrogen treated plants, standard *Rhizobium* and negative treatment (plants without nitrogen sources and inoculation) respectively. The application of nitrogen had significantly on nodule color (could not be pink), and nodule number, nodule dry weight and nodule fresh weight (0.00 mg plant-1) (Table 1) and this indicates the negative effect of nitrogen fertilizer application on nodulation of the legume plants (Crews *et al*., 2004).

In this experiment, application of mineral nitrogen fertilizer did not improve growth and development of plants; instead it delayed and inhibited nodulation and effectiveness of nitrogen fixation potential of *Rhizobium* isolates. Chemining'wa *et al*. (2004) report reveals that addition of nitrogen fertilizer had a negative effect on the nodulation and nitrogen fixation of *Rhizobium* isolates. In Ethiopia, similar application of inorganic nitrogen fertilizer did not show any significant yield difference on lentil (Angaw and Asnakew, 1994).

Table 1. Nodulation and relative effectiveness of nitrogen fixation of *R. leguminosarum bv. viciae* isolates of North Gondar (Chilga) and South Gondar (Farta and Lay Gayint) tested on Adet faba bean variety on sand using pot experiment under greenhouse condition.

Isolates	NC	NN p^{-1}	NDM $(mg)p^{-1}$	NFW $(mg)\ p^{-1}$	SDW $(g)\ p^{-1}$	SL $(cm)p^{-1}$	RB $(g)p^{-1}$	PTN (%)	SE (%)
CHI-10	P	82.00cdefg	100.00bcdef	173.7bcde	4.693c	43.67bc	1.695cdefgh	3.639j	93.33c
CHI-2	P	70.33bcde	91.67bcde	149.0bcd	2.257ab	46.67cde	0.73 ab	3.051f	45.00ab
CHI-3	P	89.67efgh	94.00bcde	150.0bcd	6.389efg	46.00cde	3.400i	3.364h	127.00def
CHI-7	P	94.00fgh	110.67ef	231.0ef	5.158cde	43.67bc	1.652bcdefg	3.671k	102.67cd
CHI-8	P	69.00bcd	83.33b	162.0bcd	4.453c	43.67bc	1.197bcdef	2.969e	87.67c

CHI-9	P	95.67gh	105.67def	141.0bc	6.970g	47.67de	2.190gh	4.160m	138.33f
KD-1	P	90.00fgh	117.33f	265.3f	5.427cde	42.00b	1.853efgh	3.419i	110.33cdef
KD-13	P	61.33b	83.67b	116.0b	4.644c	43.33bc	1.902efgh	2.848c	92.33c
KD-19	P	63.67bc	88.33bcd	140.3bc	2.735 b	46.00cde	0.817abcd	4.111 l	54.33b
KD-4	P	88.00defgh	102.00bcdef	149.0bcd	6.808fg	48.67e	1.753defgh	4.292n	135.67ef
KD-9	P	64.67bc	85.67bc	149.0bcd	2.871b	43.67bc	0.983abcde	3.359h	56.67b
LG-1	P	103.00h	104.33cdef	153.0bcd	5.747cdefg	48.67e	2.437gh	2.760b	114.33cdef
LG-16	P	86.33defgh	101.00bcdef	186.0cde	6.481efg	43.00bc	2.647hi	3.200g	129.00def
LG-17	P	69.33bcd	99.00bcdef	186.7cde	5.496cdef	44.33bcd	0.758abc	3.677k	109.00cde
LG-9	P	76.00bcdef	105.67def	208.3def	5.535cdef	46.00cde	1.983fgh	2.925d	110.33cdef
Standard	P	81.33cdefg	90.67bcd	200.0cdef	6.290defg	47.67de	2.020fgh	3.029f	124.67def
N+		0.00a	0.00a	0.00a	5.040cd	43.33bc	1.070bcdef	2.947de	100.00cd
N-		0.00a	0.00a	0.00a	1.230a	22.00a	0.080a	0.612a	24.33a
Grand Mean		71.4	86.8	153.4	4.901	43.89	1.621	3.2241	97.5
CV (%)		16.4	13.7	26.1	14.4	4.6	30.7	0.5	15.5
LSD (0.05)		19.37	19.70	66.28	1.1693	3.312	0.8238	0.0277	25.05

P= pink, NC= nodule color, NN= nodule number, NDW= nodule dry weight, NFW= nodule fresh weight, SDW= shoot dry weight, SL=shoot length, RB= root biomass, PTN=plant total nitrogen, SE=symbiotic effectiveness, p^{-1}=per plant. N- = without chemical and inoculation, N+ =with optimum amount of N fertilizer, CV= Coefficient of variation, LSD= least significant difference. 0 = not found. Means within a column followed by the same letters are not significant at $p< 0.05$.

Based on the percentage differences of shoot dry weight of inoculated and nitrogen-fertilized plants a measure of effectiveness in Purcinho (2002), more than 80% of the isolates were found to be highly effective which were greater than 80%, 13 % were effective and 7 % was lowly effective nitrogen fixers (Table 1). The highest scores of 87.67 % to 138.33 % effectiveness were displayed by isolates CHI-3, CHI-7, CHI-8, CHI-9 and CHI-10 from Chilga, KD-4 and KD-13 from Kimir Dingay and LG-1, LG-9, LG-16 and LG-17 from Lay Gayint areas (Table 1). The data showed that, more than 93% of the rhizobial isolates from North and South Gondar were highly effective and effective *Rhizobium* on the sand culture. This result reveals that, the effectiveness of our isolates were relatively high with the finding of Zerihun and Fassil, (2011) where 80% of the isolates from North Gondar were effective, but contrary to the previous finding of Desta and Angaw, (1987), only 11% of the isolates from Central Shewa were effective. According to Van Berkum *et al*., (1995) and Brockman and Bezdicek, (1989) report, variation in effectiveness of isolates was also found to be widespread in Ethiopia and USA respectively.

According to Graham O'Hara *et al*. (2002) report, the first parameter for a *Rhizobium* used as inoculant or biofertilizer is, it must be superior and highly effective in nitrogen fixing ability

forming symbiotic association with the host legume. The best nine isolates with Adet faba bean variety showed effectiveness ranging from 102.7% to 138.3% as compared to the nitrogen treated plants (Table 1). More highly effectiveness of isolates indicates the fact that the production of plant growth promoting hormone (Erum and Bano, 2008). Also the best four isolates (CHI-3, CHI-9, KD-4, and LG-16) showed effectiveness of 127%, 138.33%, 135.67% and 129% respectively as compared to inoculants of standard *Rhizobium* which showed effectiveness of 124.67%. The standard *Rhizobium* inoculated plants also showed effectiveness over nitrogen treated plants (Table 1).

In this study, more highly effective isolates were obtained compared to other investigator reports. According to Dereje *et al.* (2015) finding 56% the isolates were highly effective in both Degaga and Dosha varieties collected from acidic soils of Ethiopia, Girmaye *et al.,* (2014) reported that 16% of the isolates of faba bean were highly effective collected from acidic soils of Wollega, Western Ethiopia and Anteneh, (2012) result showed that 20.9% of isolates were very effective collected from major lentil growing areas of Ethiopia. Generally, the results of this study indicates that, screening of local *Rhizobium* isolates gives paramount importance for enhancement of dinitrogen fixation in faba bean.

Correlation analysis

Nodule number was found to be positively correlated with nodule fresh weight ($r= 0.469$ $P <0.01$) and strongly positive correlated with nodule dry mass ($r=0.677$, $P <0.01$), shoot dry weight ($r = 0.591$, $P <0.01$), root biomass ($r=0.561$, $P <0.01$) and symbiotic effectiveness ($r=0.586$, $P <0.01$) (Table 2). Shoot dry weight was found to be positively correlated with nodule dry mass ($r=0.393$, $P <0.01$), strongly positive correlated with root biomass($r=0.614$, $P <0.01$) and symbiotic effectiveness($r= 0.994$, $P <0.01$) (Table 2). Shoot dry weight and nodule dry weights are usually positively correlated and Somasegaran and Hoben (1994) finding reveals that, shoot dry weight was used regularly as an indicator of relative symbiotic effectiveness.

Shoot length was found to be positively correlated with nodule dry mass ($r= 0.396$, $P <0.01$) and with nodule fresh weight ($r= 0.391$, $P <0.01$) (Table 2). Nodule fresh weight was negatively correlated with soil nitrogen content ($r=-0.318$, $P <0.05$) and there was also

strongly positive correlation among nodule fresh weight (r=0.775, P <0.01), root biomass (r=0.610, P <0.01) (Table 2). A similar result was reported on lentil, using growth pot experiment by Anteneh (2012) and groundnut (Nguyen *et al*., 2002). Many research findings reveal that nodulation status positively correlated with plant tissue nitrogen, symbiotic effectiveness and shoot biomass or dry weight (Mnalku *et al*., 2009; Atici *et al*., 2005).

Table 2. Correlation coefficients among investigated parameters in faba bean.

	NN p^{-1}	NDM (mg) p^{-1}	NFW (mg) p^{-1}	SDW(g) p^{-1}	SL (g) p^{-1}	RB (g) p^{-1}	PTN (%)	SE (%)	Soil pH	Soil N (%)
NN p^{-1}	1	0.677**	0.469**	0.591**	0.136ns	0.561**	0.115ns	0.586**	0.106ns	-0.285ns
NDM (g)p^{-1}	0.677**	1	0.775**	0.393**	0.396**	0.300*	0.158ns	0.416**	-0.055ns	-0.270ns
NFW (g) p^{-1}	0.469**	0.775**	1	0.217ns	0.391**	0.099ns	0.018ns	0.239ns	-0.060ns	-0.318*
SDW (g) p^{-1}	0.591**	0.393**	0.217ns	1	0.098ns	0.614**	0.181ns	0.994**	-0.088ns	-0.205ns
SL (cm) p^{-1}	0.136ns	0.396**	0.391**	0.098ns	1	0.124ns	0.190ns	0.082ns	0.070ns	-0.081ns
RB (g) p^{-1}	0.561**	0.300*	0.099ns	0.614**	0.124ns	1	0.144ns	0.610**	-0.053ns	-0.236ns
PTN (%)	0.115ns	0.158ns	0.018ns	0.181ns	0.190ns	0.144ns	1	0.178ns	0.284ns	0.202ns
SE (%)	0.586**	0.416**	0.239ns	0.994**	0.082ns	0.610**	0.178ns	1	-0.095ns	-0.202n
Soil pH	0.106ns	-0.055ns	-0.060ns	-0.088ns	0.070ns	-0.053ns	0.284ns	-0.095ns	1	-0.410**
Soil N (%)	-0.285ns	-0.270ns	-0.318*	-0.205ns	-0.081ns	-0.236ns	0.202ns	-0.202ns	-0.410**	1

** = significant at P < 0.01, * = significant at P < 0.05 and ns = not significant at p <0.05
NN=nodule number, NDM (g)=nodule dry mass, NFW (g)=nodule fresh weight, SDW (g)=shoot dry weight, SL (cm)=shoot length, RB (g)=root biomass, PTN (ppm)= plant total nitrogen, SE (%)= symbiotic effectiveness, Soil N(ppm)= soil nitrogen and p^{-1}=per plant.

CONCLUSION AND RECOMMENDATION

In the present study, most of our isolates displayed abundant diversity in their response to morphological and physiological characteristics. Inoculation of isolates significantly increased at all investigated parameters such as, number of nodules per plant, nodule fresh weight, nodule dry mass, shoot dry weight, shoot length, root biomass, plant total nitrogen and symbiotic effectiveness as compared to the standard and control treatments. About 80% of the isolates collected from major faba bean growing areas of north and south Gondar, Ethiopia were found to be highly effective , 13 % were effective, only one isolate (7 %) from Chilga area categorized as lowly effective and none of isolates grouped as ineffective.

The best nine effective isolates were selected over nitrogen treated plants with Adet faba bean variety from Chilga (3), Kimir Dingay (2) and Lay Gayint (4) study areas. The best four

effective isolates, 2 of them from Chilga, 1 from Kimir Dingay and 1 from Lay Gayint were also selected as compared to the standard *Rhizobium* isolate from National Soil Laboratory, Addis Abeba.

Inoculation of selected *Rhizobium* isolates revealed shoot dry weight enhancement over nitrogen treated plants of Adet faba bean on sand culture using pot experiment under controlled greenhouse condition. Finally, further investigations of very effective isolates need to be tested under greenhouse and field condition on soil culture to assess their competitiveness ability, adaptability to the wide edaphic condition and survival and colonization within soil. Further research work with various molecular approaches should be conducted to investigate the protein and DNA pattern for better classification of the *Rhizobium* strains.

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Abundance, diversity and distribution of macrophytes in relation to water quality in the littoral zone of Lake Tana, Ethiopia

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Abstract:

Macrophytes are important components of the lake ecosystem with influencing water quality by taking up nutrients, releasing dissolved organic matter, and increasing sedimentation in the ecosystems. In view of this, understanding and quantifying the species composition and abundance of macrophytes in relation to the water quality is very vital for integrated management practices of these ecosystems. The main objective of this study was to investigate the distribution of macrophyte assemblages in relation to water quality in the littoral zone of Lake Tana. Physicochemical parameters were measured *in situ* using portable

multi-probe system and selected nutrients; NO_3-N, SRP and TP were analyzed using standard methods while macrophytes were collected using belt transect method. A total of 30 species which belonged to 15 families in the south western littoral zone and 41 species that belonged to 20 families in the north eastern littoral zone were identified in the lake. The emergent group had the highest percentage composition and attained the highest relative frequency and density, followed by floating and other forms in the south western littoral zone whereas in the north eastern zone, *E.crassipes (*Water hyacinth) was the dominant macrophyte. Relatively better diversity index was recorded in ZegieYiganda in the south western and in the Debre Sina and Rib in the north eastern littoral zones with less anthropogenic impact and minimal or no water hyacinth impact respectively. RDA showed that the density of *A. Africana, C. alopecuroides, C. demersum, C. macrostachyo and, D. milanjiana*was positively associated with SRP, pH, TP and Secchi, whereas that of *E. stagnina,* was with that of NO_3-N. In conclusion, Lake Tana shows increasing trend in concentration of nutrients and the reduction trend in transparency and water level and a shift in species composition of macrophytes towards turbidity-tolerant floating and emergent macrophyte species.

Key words: nutrient, redundancy analysis, transparency, turbidity-tolerant

1. Introduction

A study of macrophyte species composition, diversity and distribution is an essential component for understanding lake ecosystems. This is due to the important ecological role of macrophyte vegetation and the ability of the vegetation to characterize the water quality (Ciecierska and Kolada, 2014). Species composition and distribution of macrophytes in lake ecosystems depend on various environmental factors such as light, water temperature, substrate composition, disturbance and quality of the lake water (Wetzel, 2001). Macrophytes are important components of the lake ecosystem in that they influence water quality by taking up nutrients, releasing dissolved organic matter, and increasing sedimentation by absorbing turbulent energy (Ciecierska and Kolada, 2014). In view of the significant role played by macrophytes in lake ecosystems, understanding and quantifying the species composition and

abundance in relation to the nutrient dynamics is very vital for integrated management practices of these ecosystems.

2. Statement of the problem

Some of the dominant macrophyte species in Lake Tana were listed in the study of Ayalew Wondie *et al*, (2007), Imoobe and Akoma (2008), Negash Atinafu *et al*.(2011), and Ayalew Wondie and Seyoum Mengistu (2014). Historical data on macrophyte distribution pattern, abundance and diversity of the littoral zone of Lake Tana were not reported in detail in these and other similar works (e.g. Hughes and Hughes, 1992). However, Some of the recent studies in Lake Tana limnology and on hydrology have shown evidence of some undesirable changes that have occurred in the lake, such as an increase in net annual loss of water from the lake as evaporation losses during October to June exceed input via rainfall (Molla, and Menelik, 2004), increasing trend of sedimentation and silt load (Ayalew Wondie *et al*., 2007).

However, diversity of aquatic macrophytes with respect to distribution, occurrence and species composition in relation to the current nutrient dynamics of the lake water has not been investigated in detail so far. The area coverage of the weed has been estimated to be 20,000 hectares in 2012 and in the reports of Wassie Anteneh *et al*. (2014), it has been stated that the weed seems replacing the indigenous macrophyte, *Cyperus papyrus* in the north eastern shore of the lake hence, needs further study to know the real status of the weed relative to the indigenous macrophyte species in the lake to produce basic data for its proper management.

The aquatic macrophytes of Lake Tana are among the least understood and least studied components of the Lake biota. Therefore, this study was undertaken to assess the status of occurrence and species composition of macrophytes in relation to physico-chemical factors and their dynamics in the littoral zone and to investigate percentage coverage and distribution of the invasive weed, *Eichhornia crassipes* (water hyacinth) and its impact on diversity and composition of other macrophytes in the lake.

3. Objective of the Study:

The main objective of this study was to investigate the distribution of macrophyte assemblages in relation to water quality in the littoral zone of Lake Tana and to investigate the impact of the invasive weed, *Eichhornia crassipes* (water hyacinth) on diversity and composition of other macrophytes in the lake.

4. Materials and methods

4.1 Study area: Lake Tana is the largest fresh water body in Ethiopia, located at an altitude of 1786 meter above sea level. The main tributaries to the lake are Gilgel Abbay, Megech River, Gumara River and the Rib River (Fig.1).

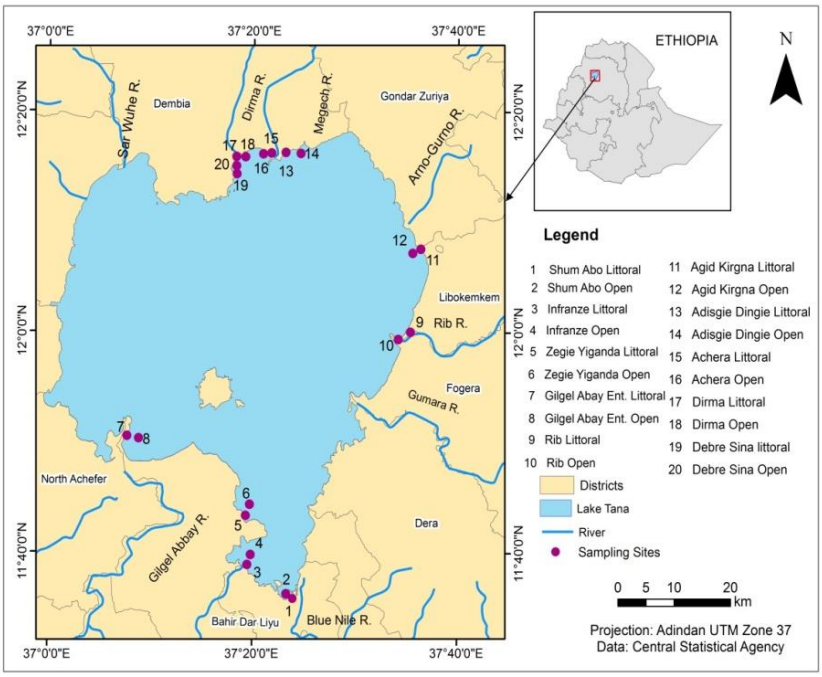

Fig.1 Location map of sampling sites in Lake Tana

4.2. Sampling design: Water for physico-chemical variables and macrophyte samples were collected from twenty purposely selected sampling points in south western and north eastern side of the lake; Shum Abo, Infranz, Zegie Yigandaj, Gilgel Abay, Rib, Dirma, Agid Kirgna, Adisgie Dingie, Achera, and Debre Sina both in open and littoral sites (Fig.1) for the study period of 2013 – September 2014.

4.2.1 Macrophyte sampling, identification and quantitative studies

Macrophytes were collected manually from all the study sites of the littoral zone of the lake. After collection, the macrophyte samples were rinsed *in situ*, blotted, pressed and transported

to the National Herbarium, Addis Ababa University, Ethiopia. To analyze the macrophyte community, a belt transect method was employed as recommended by IEP (2009). The relative frequency and relative density of each species were calculated as in the study by Singh *et al.* (2013).

4.2.2. Physicochemical parameters: Physicochemical parameters: DO, pH, EC, TDS and temperature were measured *in situ* using a YSI 556 multi-probe systems while Secchi was measured by lowering a 20 cm diameter circular disc (Secchi disc) into the water column. Water samples were also collected to analyze for NO_3-N, SRP and TP at each sampling visit using standard procedures as indicated in APHA (1999).

4.3. Data analysis: The relationship between macrophyte species abundance and physicochemical variables was evaluated by redundancy analysis (RDA) and CCA using CANOCO for windows 4.5 according to Leps and Smilauer (1999). Macrophyte species diversity in the lake was computed using Shannon and Weiner Diversity Index following Shannon and Weiner (1963).

5. Results

5.1. Species composition, abundance and distribution of macrophytes in south western littoral zone of the lake

A total of thirty macrophyte species, which belonged to fifteen families, were identified, and their relative frequency and density were determined (Appendix 1). The percentage compositions of the different macrophytic species were found maximum in the emergent group (83%), which was then followed, by the submerged species and rooted floating (each account 7%). The lowest percentage (3%) was contributed by free-floating species. The H′ value and evenness index for the four sampling sites followed the order as ZegieYiganda site > Shum Abo site > GilgelAbay entrance site > Infranz site (Table 1).

Table 1 Spatial Shannon Weiner diversity index H' and Evenness Value of South Western littoral zone of Lake Tana macrophytes, n= 4

Name of site	Site Characteristics	Number of species	Shannon Weiner diversity index H` Value	Evenness
Site 1 SAL	Near to Shum-Abo Resort	22	2.48	0.54
Site 3 INL	Close to human settlements and some fisheries activity	18	1.86	0.36
Site 5 ZEL	Better protected from anthropogenic impacts	22	**2.63**	**0.63**
Site 7 GAL	At the entrance of GilgelAbay River	19	2.38	0.57

5.2. Changes and characteristics of physico-chemical parameters in south western littoral zone of the lake

Relatively more variation in mean values of physical-chemical parameters among sites was observed for EC, TP, TDS and depth of the lake than for other parameters (Table 2) Nutrients (SRP and NO_3-N) were significantly higher in the littoral sites than open water zone ($p < 0.05$, paired - samples t-test).

Table 2 Characteristics of physico-chemical variables of the study sites in the south western littoral zone of Lake Tana (Mean±S.D) (n=4

Site	DO (mg/L)	pH (Range)	EC (µS cm^{-1})	TDS (mg/L)	Temp (0c)	Sec. (m)	Depth (m)	Nitrate (mg/L)	SRP (mg/L)	TP (mg/L)
SAO	6.89±0.49	7.61-8.92	161.70± 7.24	101.20± 3.85	24.32±0.67	0.68±0.10	5.14±0.52	0.50±0.09	0.22±0.06	0.59±3.93
SAL	6.81± 0.75	8.92± 0.27	164.18± 7.19	101.73± 2.97	25.52±0.97	0.63±0.11	2.60±0.38	1.10±0.56	0.34±0.11	1.00±5.09
INO	6.85± 0.57	8.43± 0.39	160.60± 5.63	99.63± 2.79	24.15±0.70	0.45±0.15	5.57±0.46	0.81±0.31	0.23±0.09	0.73±3.27
INL	6.41± 0.58	8.14± 0.35	163.40± 6.81	101.88± 3.69	25.18±0.92	0.56±0.11	2.35±0.37	1.53±0.35	0.26±0.05	0.53±2.07
GAO	6.72± 0.37	8.31± 0.37	155.13± 9.55	97.23± 4.80	24.53±0.61	0.46±0.16	4.83±1.28	1.00±0.48	0.15±0.05	0.60±2.77
GAL	6.65± 0.92	7.61± 0.15	152.48±17.90	94.95± 10.05	24.50±0.93	0.29±0.08	2.05±0.42	1.83±0.91	0.22±0.04	0.53±2.06
ZEO	7.02± 0.43	8.54± 0.16	156.45±5.25	97.95± 2.91	25.05±0.30	0.61±0.16	6.37±0.64	0.89±0.53	0.15±0.04	0.22±0.40
ZEL	7.54± 1.00	8.50± 0.32	162.88±7.77	101.70± 4.34	26.34±0.39	0.48±0.10	1.28±0.23	1.09±0.65	0.28±0.04	0.37±0.92

5.3. Relationship between physico-chemical parameters and macrophyte density in the south western littoral zone of the lake

The results of the redundancy analysis (RDA) between water quality data and macrophyte data showed the first two axes make 75.3% of the cumulative percentage of variance in species–environmental relationship. The first axis, which contributed 39.9% of the variance, was positively and strongly correlated with TP, SRP, pH and Secchi depth. TDS and EC showed the strongest and positive correlations with axes 2 (Fig. 2).

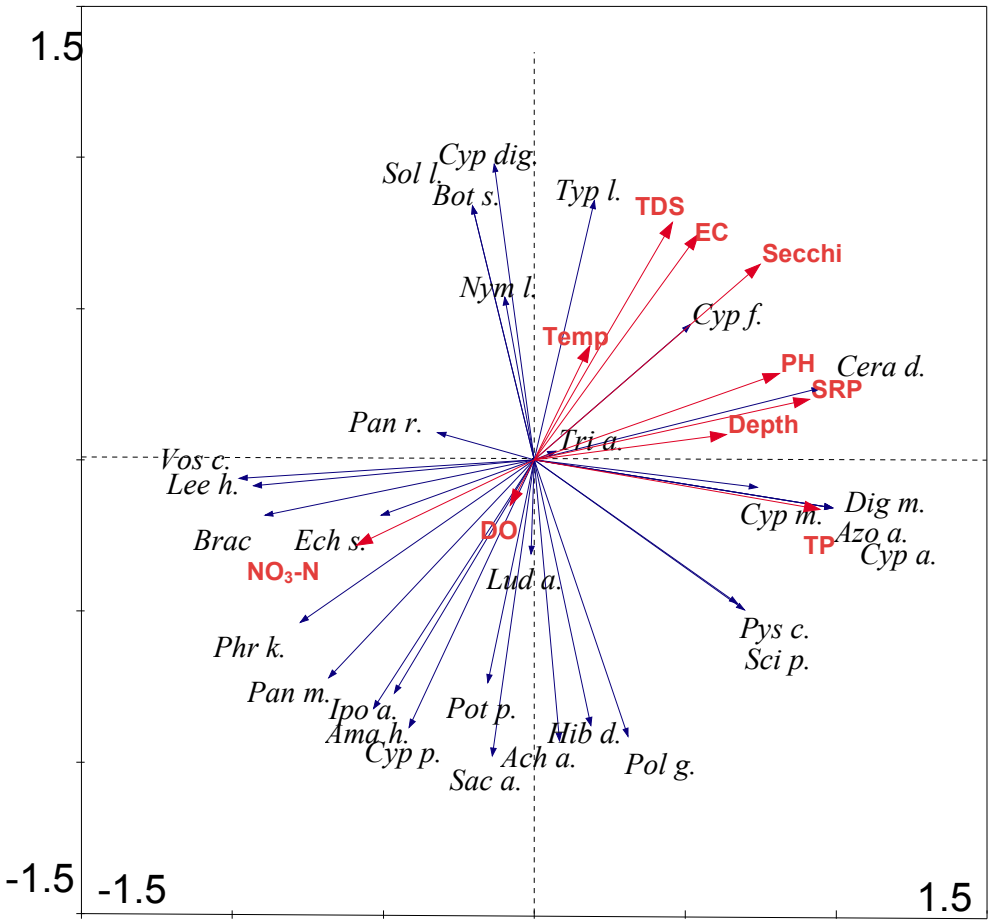

Fig.2 Plot of the first two axes of the redundancy analysis (RDA) for macrophyte species and physicochemical variables in the south western littoral zone

The density of *Azolla africana*, *Cyperus alopecuroides, Ceratophylum demersum, Cyperus macrostachyos, Digitaria milanjiana, Pyscnochys coerulea* and *Scirpus pallidus* was positively and strongly associated with SRP, pH, TP and Secchi depth (Fig.2). The density of *Vossia cuspidata, Brachiaria sp. and Leersia hexandra* was also negatively and strongly

associated with SRP and pH whereas the density of *Typha latifolia* was positively and strongly associated with TDS and EC (Fig.2).

5.4 Percentage coverage and distribution of *Eichhornia crassipes* (water hyacinth) and its impact on diversity and composition of macrophytes in the north eastern littoral zone of the lake

A total of 41 macrophyte species belonged to 20 families were identified in the North Eastern littoral zone of Lake Tana, and their relative frequency and density were presented (App. 2). *Eichhornia crassipes (*Water hyacinth) is the dominant macrophyte in most of the sampling sites that attained the highest relative frequency and density. Among these identified species water hyacinth accounted the dominant coverage (89.86%, 56.09%, 46.71% and 34.83% in Agidkirgna, Achera, Adisgie Dingie and Dirma sampling sites respectively).Whereas the non-infested sampling site Rib and minimal infested site. Debresina were dominated by *Echinochloa stagnina (33.66%)* and *Cyperus papyrus* (12.51%) respectively.

In this study mean macrophyte species diversity of north eastern littoral zone of Lake Tana was ranged from 1.3 - 2.99 where higher H′ values are observed in minimal infested and non-infested sampling sites; in DS and in RB sampling sites while the lowest H′ value was in one of the water hyacinth infested site, AK sampling site. Comparatively, all the water hyacinth infested sites have lower H′ values than none and minimal water hyacinth infested sites (Table 3). Similarly, the highest evenness index was in the minimal infested site, DR sampling site and its lower value is observed in the water hyacinth infested site, AC sampling site (Table 3).

Table 3 Macrophyte diversity index of the north eastern littoral zone of Lake Tana

Parameer	Non-infested	Infested			Minimal infested	
	RB	AK	AD	AC	DR	DS
No.ofspp	15	8	10	9	7	29
H`value	2.11	1.36	1.56	1.36	1.75	2.99
Evenness value	0.55	0.49	0.47	0.43	0.82	0.69

5.5 Physicochemical characteristics of the water quality in the north eastern zone

More variation in mean values of physical-chemical parameters among sites was observed for DO, TDS Temperature and depth of the lake and for almost all nutrients in water (Table 4).

Table 4 Mean Physico-chemical characteristics of the water quality in the north eastern littoral zone of Lake Tana (Mean ± S.D) (n=8)

Site	DO mg/L	pH(Range)	EC(µs/cm)	TDS mg/L	Temp.(0c)	Secchi.(m)	Depth(m)	NO_3-N mg/L	SRP mg/L	TP mg/L	Sampling site status
RO	5.75±0.30	7.91- 8.70	170.50±16.66	100.75±1.49	22.71±0.45	0.33±0.04	**6.65±0.85**	0.42±0.10	0.14±0.05	0.81±3.93	**Non-Infested**
RL	6.32±1.07	8.16±0.22	**225.00±41.63**	116.00±6.01	22.87±2.10	0.23±0.05	**0.36±0.09**	0.79±0.15	0.47±0.18	1.20±1.57	
AKO	5.78±0.15	8.29±0.38	156.50±2.02	103.25±2.17	23.50±0.82	0.31±0.05	3.52±0.57	**0.28±0.11**	0.17±0.06	0.70±3.61	**Infested**
AKL	**5.19±0.17**	8.07±0.04	165.00±6.84	**127.75±24.77**	**25.72±0.75**	**0.18±0.03**	0.93±0.33	0.91±0.27	0.31±0.09	**2.06±8.32**	
ADO	6.37±0.22	8.29±0.53	**142.00±9.94**	93.75±4.89	22.88±0.58	**0.42±0.05**	4.38±0.59	0.46±0.10	0.11±0.04	0.51±2.18	
ADL	7.03±1.12	8.12±0.20	151.75±8.32	98.50±5.20	**20.86±1.56**	0.24±0.04	0.69±0.18	0.96±0.28	0.32±0.06	0.81±3.47	
ACO	6.84±0.55	8.39±0.26	148.50±4.17	96.00±3.34	23.17±0.64	0.31±0.07	2.90±0.26	0.79±0.22	0.16±0.03	**0.44±2.13**	
ACL	5.81±0.39	7.96±0.28	156.25±5.75	101.75±3.82	23.48±0.79	0.28±0.08	0.94±0.14	1.43±0.45	0.20±0.04	0.55±1.85	
DRO	**7.43±1.00**	**8.70±0.36**	144.75±6.09	93.75±4.42	22.95±0.78	0.38±0.09	5.31±2.33	0.58±0.14	0.19±0.06	0.80±3.23	**Minimal infested**
DRL	7.15±0.68	8.07±0.35	150.75±5.31	98.00±3.54	23.88±0.81	0.25±0.04	1.15±0.25	**1.56±0.70**	0.38±0.15	1.07±3.64	
DSO	7.17±0.39	8.67±0.48	144.75±6.37	**93.25±4.87**	22.91±0.65	0.40±0.06	4.31±0.18	0.58±0.17	0.15±0.09	0.51±1.40	
DSL	7.09±0.69	**7.91±0.67**	147.25±5.50	95.75±3.57	23.04±0.73	0.36±0.04	1.99±0.37	1.32±0.58	0.35±0.09	0.62±2.92	

5.6 Relationship between physicochemical parameters and density of macrophyte species in the north eastern zone

Results of canonical correspondence analysis (CCA) showed that the first two axes make 64.6% of the cumulative percentage of variance in species–environmental relationship. The first axis, which contributed 34.5% of the variance, was positively and strongly correlated with EC and pH and depth of the water level and NO_3-N were negatively but strongly correlated to the axis (Fig. 3).

From the analysis, the density of *Brachiaria* sp.,*Eichhornia crassipes, Echinochloa pyramidalis, Echinochloa crus-galli and Nymphoides indica, w*as positively associated with temperature, *Nymphaea lotus, Phragmites karka, Ludwigia abysinica* and *Ipomoea aquatica*

was positively associated with nitrate, however, negatively to SRP and EC (Fig. 3). In contrast, positive associations to these parameters were observed with that of *Glinus lotoides, Triumfetta annua.* These species are also positively associated with pH but negatively to nitrate (Fig. 3).

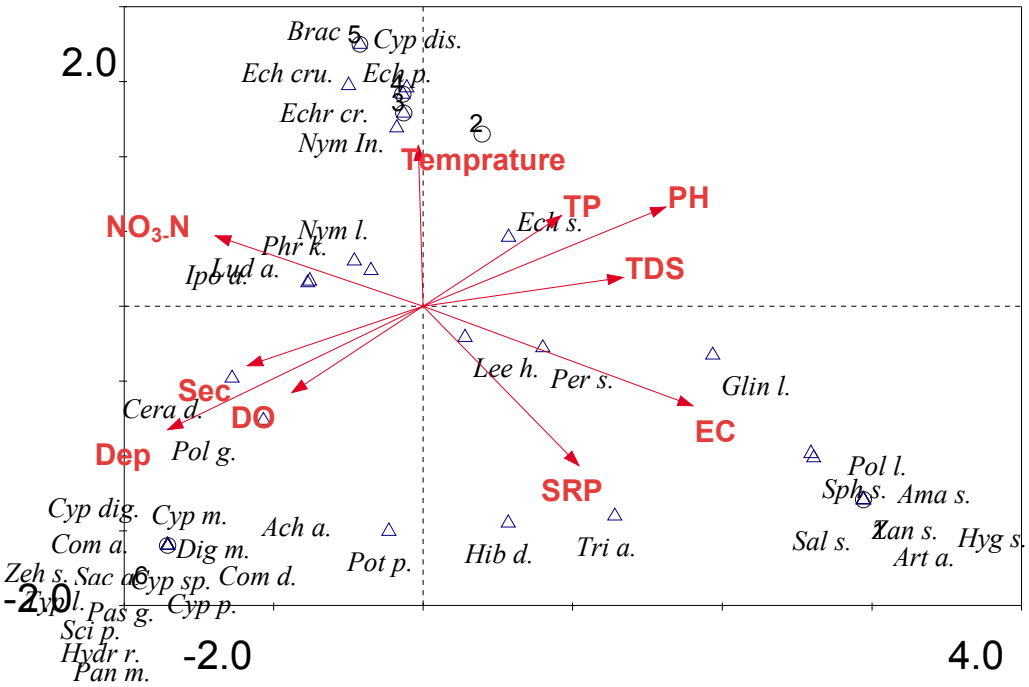

Fig. 3 Plot of the Canonical Correspondence Analysis (CCA) for macrophyte species and physicochemical variables in the north eastern littoral zone

6. Discussion

6.1. Macrophyte species diversity in Lake Tana

The highest macrophyte species diversity index (H′) and evenness index values was observed in sites; Zegie Yiganda littoral, Debre Sina littoral and Dirma littoral which are better protected from anthropogenic impacts (not threatened by industrial and urban expansions and forest clearing and was also non or minimal impacted from water hyacinth.). Lower diversity index (H′) and evenness index values were observed in Infranz littoral , Agid Kirgna littoral and Achera littoral sites where Infranz littoral site being close to human settlements higher anthropogenic impacts are expected to cause a decrease species diversity and composition.

Besides, Agid Kirgna and Achera sites are also the highly water hyacinth infested and impacted sites, consequently could have low diversity of macrophytes.

The macrophyte species diversity of Lake Tana is low compared with some published data of similar works on tropical shallow lakes and reservoirs (e.g. Burlakoti and Karmacharya, 2004). Low diversity is expected in lakes that have an outflow, because accumulated nutrients produced from different sources could be flushed out (Burlakoti and Karmacharya, 2004; Girum Tamire and Seyoum Mengistu, 2012). Lake Tana is becoming highly turbid; its Secchi depth reading decreased considerably (18 -68 cm) as compared to the previous situation, and as a result, the growth of many submerged species is limited. The lake has also a shorter water retention time (about 3 years) (Seifu Kebede *et al*., 2006), and establishment of new macrophytes could be hindered.

6.2. Emergent macrophyte and Water hyacinth dominance in Lake Tana

Lake Tana is highly dominated by emergent macrophytes and Water hyacinth, which could be due to their high tolerance for turbidity and water-level fluctuation (Nurminen, 2003) and increasing trend in nutrient concentration as well.

Most of the natural vegetation could be destroyed for farm land expansion (Recession farming), for settlement and intensive grazing. The impact of water hyacinth on the growth of other macrophytes species has also noticeable effect. This effect has been reported by different studies. For instance, the decimation effect of water hyacinth on the floating leafed *Nymphaea lotus* and the submerged plants, *Ceratophyllum demersum* has been reported by (Aloo *et al.*, 2013) in Lake Victoria, Kenya.

7. Conclusion and Recommendations

The study found that aquatic macrophyte species abundance and diversity of Lake Tana was low compared to some other water bodies where similar studies were carried out. The decreasing trend in water level and transparency of the lake and increasing trend of nutrients like nitrate and SRP levels have contributed to this conspicuous shift in macrophyte composition and reduction in abundance of some macrophytes in the lake. Lake Tana has higher concentration of SRP compared to some Ethiopian lakes (e.g. Lake Chamo, Abaya and

Ziway). *Azolla africana*, *Cyperus alopecuroides, Ceratophyllum demersum, Cyperus macrostachyos and Digitaria milanjiana,* were almost restricted to sites where there was higher SRP and TP and *Echinochloa stagnina*, *Brachiaria sp.* and *Phragmites karka*, was to sites where there was higher nitrate concentration and they were among the macrophyte species that indicate high nutrient levels (SRP, TP and nitrate) of lake water.

Therefore, further studies on determination of key species of macrophytes performing vital functions such as filters of pollutants and silt, and indicators of environmental change in the lake is very vital. Sediment load might have great effects on macrophyte composition; studies on internal and external nutrient load dynamics of the lake should be done. It is high time to look into the problems of the lake caused due to impact water hyacinth therefore studies for best practices of water hyacinth control are needed.

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Appendix 1 Macrophyte Species identified and their relative frequency and density in the south western littoral zone of Lake Tana

Species	Family	Lifeforms	Relative Frequency (%)	Relative Density (%)
Achyranthes aspera L.	Amaranthaceae	Emergent	3.17	0.94
Amaranthus hybridus L.	Amaranthaceae	Emergent	6.35	1.7
Azolla africana Desv.	Azollaceae	Free floating	0.79	8.49
Bothriocline schimperi Olivo&Hiern ex Benth	Asteraceae	Emergent	0.79	0.47
Brachiaria sp.	Poaceae	Emergent	3.57	1.13
Ceratophyllum demersum L.	Ceratophyllaceae	Sumerged	6.75	5.19
Cyperus alopecuroides Rotb.	Cyperaceae	Emergent	1.59	0.57
Cyperus digitatus Roxb.	Cyperaceae	Emergent	3.57	18.87
Cyperus fischerianus A. Rich	Cyperaceae	Emergent	4.37	2.08
Cyperus macrostachyos Lam.	Cyperaceae	Emergent	0.79	1.6
Cyperus papyrus L.	Cyperaceae	Emergent	7.54	11.89
Digitaria milanjiana (Rendle) Stapf	Poaceae	Emergent	1.19	0.57
Echinochloa stagnina (Retz.) P. Beauv.	Poaceae	Emergent	1.19	2.26
Hibiscus diversifolius Jacq	Malvaceae	Emergent	2.38	0.57
Ipomoea aquatica (L.) Sweet	Convolvulaceae	Rooted floating	1.19	0.57
Leersia hexandra SW.	Poaceae	Emergent	3.57	1.23
Ludwigia abysinica A. Ric	Onagraceae	Emergent	6.35	3.68
Nymphaea lotus L.	Nymphaeaceae	Rooted floating	1.98	0.57
Panicum repens L.	Poaceae	Emergent	0.4	0.47
Panicum maximum Jacq	Poaceae	Emergent	4.76	2.08
Phragmites karka (Retz.) Trin. exSteud.	Poaceae	Emergent	9.13	15.94
Polygonum glabrum Willd	Polygonaceae	Emergent	5.16	3.02
Potomagton pectinatus L.	Potamogetonaceae	Submerged	4.76	1.89

Pyscnochys coerulea Hook	Lamiaceae	Emergent	1.19	1.04
Sacciolepis africana C.E.Hubb.&Snoden	Poaceae	Emergent	3.17	1.89
Scirpus pallidus (Britton) Fernald	Cyperaceae	Emergent	1.59	0.75
Solenostemon latifolius (Benth) J.K Morton	Lamiaceae	Emergent	0.4	0.09
Triumfetta annua L.	Tiliaceae	Emergent	1.98	0.75
Typha latifolia L.	Typhaceae	Emergent	6.35	7.08
Vossia cuspidata (Roxb.) Griff.	Poaceae	Emergent	3.97	2.64

Appendix 2 Macrophyte species identified and their relative frequency and density on sampling sites with varied water hyacinth infestation level in the north eastern littoral zone of Lake Tana

spp	Family	Non-infested		Infested						Minimal infested			
		RB		AK		AD		AC		DR		DS	
		rel-fr	rel-d	rel-fr	rel-d	rel-fr	rel-d	rel-fr	rel-d	rel-fr	rel-d	rel-fr	rel-d
Achyranthes aspera L	*Amaranthaceae*	-	-	-	-	-	-	-	-	-	-	3.31	0.81
Amaranthus spinosus L	*Amaranthaceae*	3.95	1.56	-	-	-	-	-	-	-	-	-	-
Artemisia absinthium L	*Asteraceae*	3.95	4.3	-	-	-	-	-	-	-	-	-	-
Brachiaria sp.	Poaceae	-	-	-	-	-	-	-	-	15.48	8.06	-	-
Ceratophyllum demersum L	Ceratophyllaceae	-	-	-	-	-	-	-	-	3.57	2.78	9.52	13.14
*Commelinadiffusa*Burm.f.	*Commelinaceae*	-	-	-	-	-	-	-	-	-	-	4.35	2.59
Commolina africana L	*Commelinaceae*	-	-	-	-	-	-	-	-	-	-	2	1.12
Cyperus digitatus Roxb.	Cyperaceae	-	-	-	-	-	-	-	-	-	-	2.25	1.63
*Cyperus distans*L.f.	Cyperaceae	-	-	-	-	-	-	2.78	2.8	-	-	-	-
Cyperus macrostachyos Lam.	Cyperaceae	-	-	-	-	-	-	-	-	-	-	0.93	0.46
Cyperus papyrus L.	Cyperaceae	-	-	-	-	-	-	-	-	-	-	3.56	8.15
Cyperus sp.	Cyperaceae	-	-	-	-	-	-	-	-	-	-	3.93	8.4
Digitaria milanjiana (Rendle) Stapf	*Poaceae*	-	-	-	-	-	-	-	-	-	-	2.64	1.66
Echinochloa crus-galli (L.) Beauv	*Poaceae*	-	-	-	-	2.5	0.22	-	-	17.86	17.22	1	0.28
Echinochloa pyramidalis (Lam.) Hitchc. & Chase	*Poaceae*	-	-	13.17	6.49	8.75	3.22	8.43	1.44	20.24	21.11	-	-

Echinochloa stagnina (Retz.) P. Beauv.	*Poaceae*	11.11	28.68	29.11	33.87	29.17	30.13	29.32	24.99	0	0	2.18	2.29
Eichhornia crassipes (Mart.) Solms.	Pontederiaceae	-	-	20.78	47.53	22.92	51.29	29.32	63.15	11.9	32.78	1.85	2.08
Glinus lotoides L.	Molluginaceae	3.95	10.55	2.78	3.95	-	-	-	-	-	-	-	-
Hibiscus diversifolius Jacq	Malvaceae	4.86	0.98	-	-	-	-	-	-	-	-	3.31	1.04
Hydrocotyle ranunculoides L. f.	*Apiaceae*	-	-	-	-	-	-	-	-	-	-	1	1.12
Hygrophilia schulli (Hamitt) &Almeide	*Acanthaceae*	2.78	1.56	-	-	-	-	-	-	-	-	-	
Ipomoea aquatica (L.) Sweet	*Convolvulaceae*	-	-	-	-	6.25	5.3	5.95	1.73	-	-	6.02	5.52
Leersia hexandra SW.	*Poaceae*	3.4	0.59	7.32	1.92	-	-	-	-	-	-	2.85	0.98
Ludwigia abysinica A. Ric	Onagraceae	-	-	-	-	5	4.57	12.15	4.19	-	-	6.24	8.92
Nymphaea lotus L.	Nymphaeaceae	-	-	20.02	5.58	-	-	1.79	0.22	-	-	3.89	1.89
Nymphoides indica (L.) O.Kuntze	Nymphaeaceae	-	-	-	-	8.33	2.19	-	-	-	-	-	-
Panicum maximum Jacq	*Poaceae*	-	-	-	-	-	-	-	-	-	-	2.25	2.16
Paspalidium geminatum (Forssk.) Stapf	*Poaceae*	-	-	-	-	-	-	-	-	-	-	5.18	4.68
Persicaria senegalensis (Meisn.) Sojak	*Polygonaceae*	19.3	15.21	-	-	1.67	0.24	-	-	7.14	6.94	1.25	2.66
Phragmites karka (Retz.) Trin. exSteud.	*Poaceae*	6.25	0.91	4.55	0.47	13.75	2.36	8.48	1.26	23.81	11.11	10.27	12.86
Polygonum glabrum Willd	Polygonaceae	-	-	2.27	0.18	-	-	-	-	-	-	0.93	0.46
Polygonum lapathifolium L.	Polygonaceae	6.8	3.67	-	-	-	-	1.79	0.22	-	-	-	-
Potomagton pectinatus L.	Potamogetonaceae	3.4	0.7	-	-	-	-	-	-	-	-	4.81	2.12
Sacciolepis africana C.E.Hubb.&Snoden	*Poaceae*	-	-	-	-	-	-	-	-	-	-	4.43	2.92
*Salix subserrata*Willd	Salicaceae	2.78	3.13	-	-	-	-	-	-	-	-	-	-
Scirpus pallidus (Britton) Fernald	*Cyperaceae*	-	-	-	-	-	-	-	-	-	-	1	2.81

Sphaeranthus suaveolens (Forssk.) DC.	*Asteraceae*	16.59	24.33	-	-	1.67	0.48	-	-	-	-	-	-
Triumfetta annua L.	Tiliaceae	5.48	1.1	-	-	-	-	-	-	-	-	1.93	0.51
Typha latifolia L.	Trapaceae	-	-	-	-	-	-	-	-	-	-	4.95	5.96
Xanthiumum strumarium L.	Asteraceae	5.41	2.73	-	-	-	-	-	-	-	-	-	-
Zehneriascabra(L.f.) Sond.	*Cucurbtaceae*	-	-	-	-	-	-	-	-	-	-	2.18	0.76

Identification of 1BL.1RS Wheat-Rye Chromosome Translocation, Lr34 and Lr37 Rust Resistance Genes in Wheat (*Triticum aestivum* L.) Cultivars Using Molecular Markers

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Abstract

Stem rust, leaf rust and stripe rust are widespread wheat rust diseases that can cause significant yield loss in wheat production globally. The development and release of genetically disease resistant wheat cultivars is environmentally safe and effective means to control wheat rusts. Since growing cultivars carrying single rust resistance genes have favored the advent of new virulent races, pyramiding of rust resistance genes is more effective against rust. Increasing knowledge on identity and frequency rust resistance genes present in cultivars that can be used as source of resistance is a basic study. Because, using these genes in appropriate combinations in gene pyramiding can enhance the durability of resistance. The objective of this study was to assess the presence of Lr26-Yr9-Sr31 (1BL.1RS wheat-rye chromosome translocation) and the Lr37-Yr17-Sr38 and Lr34-Yr18 rust resistance genes in wheat (*Triticum aestivum* L.) cultivars. The cultivars were registered in Gene Bank of Crop Research Institute (GBCRI), Praha-Ruzyně, Czech Republic and originated from different European countries from 1992 to 2013. PCR based molecular markers, RAPD and STSs were used. According to the results, 1BL.1RS wheat-rye translocation was proved to be present in 13 (12.87%) of all cultivars tested. Lr37-Yr17-Sr38 was detected in 28 (30.7%) cultivars from the 91 registered in Czech Republic while Lr34-Yr18 was found only in one cultivar. Of all tested, five cultivars were proved to have both 1BL.1RS translocation and Lr37-Yr17-Sr38. It was able to observe that the frequency of the use of Lr37-Yr17-Sr38 in registered cultivars displays an increasing tendency. Molecular markers are efficient and ideal for identification of rust resistance genes. The information presented here will be helpful in utilization of these identified cultivars for further enhancement of genetic resistance in the respective zones. The methodology used in this study and MAS can be also applied in Ethiopia to explore rust genetic resistance in wild, cultivated and current cultivars.

Key words: Chromosome translocation, resistance genes, molecular markers, STS, RAPD

Introduction:

The stem rust, leaf rust and stripe rust which are caused by the pathogens *Pucciniagraminis* Pers. f. sp. *tritici*.; *Puccinia recondita* Rob. exDesm. f. sp. *tritici* and *Puccinia striiformis* f.sp. *tritici* respectively, are the most widespread and economically important diseases of wheat worldwide (Roelfs et al., 1992). Both in Czech Republic and Hungary, stem rusts epidemic have not been recorded in the last few decades mainly due to the use of resistant cultivars have been increased (Purnhauser et al., 2011a; Bartos, 2010). Leaf rust occurs frequently in both countries, causing higher yield losses (Bartos, 2010; Purnhauser et al., 2011). Since the last decade, yellow rust incidence has been low. The development genetically disease resistant wheat cultivar is the most widely used and effective means to control wheat rusts. Identification of resistance genes in wheat cultivars is important in the resistance breeding. These can be done by inoculating wheat accessions with known rust isolates (Knott, 1989).

Since the last few decades, genotype based selection has been increased quite rapidly (Edwards and McCouch, 2007). Molecular maps of wheat rust resistance genes/QTLs have been constructed and molecular markers have been used in marker-assisted selection (MAS). The desirable lines can be selected by marker-assisted selection on the basis of genotype rather than phenotype. A number of different types of efficient and specific molecular markers namely RAPD (Random amplified polymorphic DNA), RFLP (Restriction fragment length polymorphism), SSR (Simple sequence repeat), STS (Sequence-tagged sites) and CAPS (Cleaved amplified polymorphic sequences) have been reported for identification of wheat rust resistance gens including; Lr26/Sr31/Yr9 (1BL/1RS), Lr34/Yr18 and Lr37/Yr17/Sr38 (Mumtaz et al., 2009; Bossolini et al., 2006; Lagudah et al., 2006; Blaszczyk et al., 2005; Schnurbusch et al., 2004; Suenaga et al., 2003; Helguera et al., 2003; Nadella et al., 2002; Robert et al., 1999; De Froidmont, 1998; Nelson et al., 1997; William et al., 1997; Francis et al. 1995).

The 1BL.1RS translocations (Lr26-Yr9-Sr31) are popular wheat-rye chromosome translocations used in bread wheat breeding programs all over the world, originated from German rye cultivars 'Petkus'. A short arm of 'Petkus' rye chromosome 1RS, carries Sr31, Lr26 and Yr9 (Rabinovich, 1998; Schlegel and Korzun, 1997; Burnett et al., 1995). Although, at the beginning of its use 1BL.1RS provided good resistance against all rust species, but the leaf and yellow rust resistance was shortly overcome by new pathogen races. In the Czech Republic resistance genes on this

translocation are no longer effective to the prevailing pathotypes of leaf rust and yellow rust, only stem rust resistance remains effective (Hanzalova et al., 2009). Sr31, Sr11, Sr29, Sr36, Sr38 and Sr37 are commonly postulated stem rust resistance genes in several cultivars registered in the Czech Republic and Hungary in the last 40 years (Chrpova et al., 2012; Purnhauser et al., 2011a; Purnhauser et al., 2011b; Bartos, 2010; Csosz et al., 2007; Bartos et al., 2002).

Lr37-Yr17-Sr38 was translocated from a segment of 2NS chromosome of *Triticum ventricosum* (*Aegilopsventricosa*) to the short arm of common wheat chromosome 2AS. These 2NS/2AS translocation consists of Lr37, Sr38, and Yr17 rust resistance genes which confer resistance against all three wheat rust diseases (Helgueraet al., 2003). 2NS/2AS translocation has been extensively used in European wheat breeding programs. Lr37-Yr17-Sr38 was the most frequent adult plant leaf rust resistance gene in Czech wheat cultivars (Chrpova et al., 2012).

Lr34-Yr18 confers durable, race non-specific protection against three fungal pathogens, and has been a highly relevant gene for wheat breeding since the green revolution (Krattinger et al., 2009). The Lr26 and Lr34-Yr18 and Lr37-Yr17-Sr38 resistance genes occur frequently in Hungarian genotypes (Vida et al., 2009).

Statement of the problem: Stem rust epidemics have not been reported in the last few decades in Hungary. However, the advent of mutant races of stem rust that are virulent to the most of existing resistance genes becomes a treat to Hungary wheat production (Pretorius et al., 2000; Singh et al., 2011). Similarly, yellow rust incidence has been low in the last decade. However, this situation can be changed if yellow rust in Europe becomes adapted to higher temperatures (Chrpova et al., 2012; Bartos, 2010). Now, this possibility seems a real threat as in Hungary a new yellow rust epidemic appeared in 2013 and it was continued in a more severe form in 2014. So, continuous finding of new rust resistance gene sources should be undertaken. Moreover, cultivars that carry single rust resistance gene can favor the evolving of new virulent races. Deploying of rust resistance genes to commercial cultivars to establish durable resistance is more effective for the control of rust. Hence, increasing knowledge on identity and frequency of rust resistance genes present in cultivars that can be used as source of resistance is a basic study. Because, using these genes in appropriate combinations in gene pyramiding can enhance the

durability of resistance. The important primary step in rust resistance breeding is to find resistance sources.

Objectives: The objective of this study was to assess the presence or absence of Lr26-Yr9-Sr31 (1BL.1RS wheat-rye chromosome translocation) and the Lr37-Yr17-Sr38 and Lr34-Yr18 rust resistance genes in 101 wheat cultivars registered in Czech Republic and Hungary using PCR based molecular markers.

Materials and methods

Plant materials: One hundred one bread wheat (Triticum aestivum L.) cultivars (Table 4) were used in this study. 91 cultivars were originated from different European countries from 1992 to 2013 and registered in Gene Bank of Crop Research Institute (GBCRI), Czech Republic. The other 10 Hungarian cultivars were obtained from Cereal Non-Profit Co. Ltd. and Agricultural Research Institute of the Hungarian Academy of Sciences, Hungary. Seed was provided by Dr. Laszlo Purnhauser, (Cereal Non-Profit Co. Ltd.) and the experiment was conducted in 2014 in this institute.

DNA Isolation: Fresh leaves were taken from three 10-day-old seedlings and genomic DNA was isolated using the CTAB (cetyl-trimethyl-ammonium bromide) method (Rogers and Bendich, 1985). The extracted DNA samples were stored in deep freezer (-20^{o}C).

DNA Quantification and Dilution: To quantify the concentration of the DNA, samples and standards were run on 1.5 % agarose gel. The sample DNA band intensity was compared with that of DNA standards (5ng/µL, 10 ng/µL, 25 ng/µL, 100 ng/µL, and 200 ng/µL). The stock DNA was diluted in 0.1 M TE buffer to make 2ng/µL to use them in PCR.

PCR Amplification: One RAPD (random amplified polymorphic DNA) and two STSs (sequence tagged site) DNA markers were used. PCR amplification of genomic DNA samples with published primers (Table 1) was undertaken to identify the presence of 1BL.1RS translocation (Lr26-Yr9-Sr31), Lr34-Yr18 and Lr37-Yr17-Sr38 rust resistance genes. Thatcher cultivar and NILs containing the corresponding rust genes in Thatcher cultivar background were used as negative and positive control respectively. The PCR-mixture was prepared in 15µL reaction volume containing all necessary reagents for each primer, except the extracted DNA (Table 2). The final volume of the PCR reaction reached to 20 µL including 5 µL of extracted

DNA. The PCR was performed according to the PCR condition listed in Table 3. PCR products were amplified using the GeneAmp® PCR System 9700 (Applied Biosystems) thermal cycler.

Table 1: The resistance genes, the linked molecular marker types and name of the marker and primers sequence

Resistance genes	Marker types	Name of the marker	Sequence of primers (5'-3')	References
1BL.1RS (Lr26-Yr9-Sr31)	RAPD	OPH20	GGGAGACATC	Francis et al., 1995
Lr34-Yr18	STS	csLV34	GTTGGTTAAGACTGGTGATGG TGCTTGCTATTGCTGAATAGT	Lagudah et al., 2006
Lr37-Yr17-Sr38	STS	VENTRIUP/LN2	AGGGGCTACTGACCA AGGCT TGCAGCTACAGCAGTATGTACACAAAA	Helguera et al., 2003

Table 2: The volume and concentration of reagents used to prepare the PCR reaction for csLV34 and VENTRIUP/LN2 STS and OPH20 RAPD marker

Reagents	Stock Concentration		Reaction concentration		µL/reaction	
	csLV34 & VENTRIUP /LN2	OPH20	csLV34 & VENTRIUP /LN2	OPH20	csLV34 & VENTRIUP /LN2	OPH20
Sterile dH2O					2.3	8
PCR buffer	10x	10x	1x	1x	2.0	2
MgCl2	25 mM	-	1.5 mM	-	1.2	-
dNTP mix	1 mM	1 mM	200 µM	1 mM	4	2
Primers	0.8 pM/µM	2.5pM/µM	0.2 µM	0.35µM	5	2.8
Taq DNA polymerase	1U/µL	2U/ µL	0.5 U	0.3 U	0.5	0.15
Reaction mix in µL					15.0	15.0
Genomic DNA	2ng/µl	2ng/µl			5	5
Final PCR mix in µL					20	20

Table 3: The PCR conditions

Lr34	1BL/1RS	Lr 37
Step 1. Pre denaturation = 94^{o}C for 5 min; Step 2. Denaturation=94^{o}C for 30 sec; Step 3. Annealing= 58^{o}C for 30 sec; Step 4. Amplification= 72^{o}C for 50sec; Steps 2-4 repeated 30 times; Final extension 72 ^{o}C for 3min	Step 1. Pre denaturation = 94^{o}C for 2 min; Step 2. Denaturation =94^{o}C for 30 sec; Step 3. Annealing= 36^{o}C for 30 sec; Step 4. Amplification= 72^{o}C for 2min; Steps 2-4 repeated 40 times; Final extension 72 ^{o}C for 10min	Step 1. Pre denaturation = 94^{o}C for 2 min; Step 2. Denaturation =94^{o}C for 45 sec; Step 3. Annealing= 60^{o}C for 30 sec; Step 4. Amplification= 72^{o}C for 1min; Steps 2-4 repeated 40 times; Final extension 72 ^{o}C for 7 min

Gel electrophoresis: From each sample, 8 µL was loaded and run on 1.5 % agarose gel. GeneRuler 80 TM 100 bp DNA Ladder (Fermentas) was used as a molecular weight marker. After electrophoresis, bands were visualised under UV light (UV light transilluminator, SIGMA). Photograph of the gel were made by a digital camera for analysis. Only higher intensity, scorable and well resolved bands or fragments were scored and used for gel analysis.

Results

The presence and absence of rust resistance genes in cultivars investigated is shown in Table 4.

Table 4: Identification of leaf and stem rust resistance genes by using the specific PCR markers in wheat cultivars

No.	Cultivar	Year of Registration	Origin	1BL.1RS (Lr26-Sr3-Yr9)	Lr34-Yr18	Lr37-Yr17-Sr38
1	Alibaba	2003	Germany	0	0	0
2	Banguet	2001	Czech Republic	0	0	0
3	Bill	2001	Denmark	0	0	0
4	Brilliant	2009	Germany	1	0	1
5	Brokat	2013	Germany	0	0	0
6	Buteo	2006	Germany	0	0	0
7	Caphorn	2004	UK	0	0	0
8	Carroll	2011	Netherlands	0	0	1
9	Cimrmanova	2012	Czech Republic	0	0	0
10	Citrus	2011	Germany	0	0	0
11	Clarus	2003	UK	1	0	0
12	Clever	2001	UK	0	0	1
13	Complet	2000	Germany	0	0	1
14	Cubus	2004	Germany	0	0	0
15	Dagmar	2012	Czech Republic	0	0	0
16	Drifter	2000	Germany	0	0	1
17	Dromos	2006	Germany	0	0	0
18	Elan	2012	France	0	0	0
19	Elly	2010	Czech Republic	0	0	1
20	Etana	2013	Germany	0	0	1
21	Etana	2006	Germany	1	0	0
22	Eurofit	2006	Austria	0	0	0
23	Evina	2012	France	0	0	1
24	Fabius	2013	Austria	0	0	0
25	Fakir	2013	Germany	0	0	0
26	Federer	2009	Czech Republic	0	0	0
27	Feria	2011	France	0	0	0
28	Fermi	2011	France	0	0	0
29	Florett	2006	France	0	0	0
30	Fortis	2009	Czech Republic	0	0	0
31	Globus	2003	Germany	0	0	1
32	Golem	2011	Czech Republic	0	0	1
33	Graindor	2010	France	0	0	1
34	Hedvika	2004	Netherlands	0	0	0
35	Henrik	2010	France	0	0	0
36	Hermann	2007	France	0	0	1
37	Heroldo	2005	Germany	0	0	0
38	Hewitt	2012	Netherlands	0	0	1
39	Chevalier	2011	Germany	0	0	0
40	Iridium	2009	France	0	0	0
41	JB Asano	2012	Germany	0	0	1
42	Jindra	2010	Czech Republic	0	0	0
43	Karolinum	2003	Netherlands	1	0	0
44	Kerubino	2007	Germany	0	0	1
45	Kodex	2008	Germany	0	0	1

46	KWS Ozon	2012	Germany	0	0	0
47	Lavantus	2013	Germany	0	0	0
48	Ludwig	2000	Austria	0	0	0
49	Magister	2009	Germany	0	0	0
50	Manager	2007	Germany	0	0	1
51	Matchball	2013	Czech Republic	1	0	1
52	Matylda	2011	Czech Republic	0	0	0
53	Meritto	2003	Czech Republic	0	0	0
54	Mladka	2001	Czech Republic	0	0	0
55	Mulan	2007	Germany	0	0	0
56	Nikol	2008	Czech Republic	0	0	1
57	Orlando	2008	Denmark	1	0	1
58	Patras	2013	Germany	0	0	0
59	Pitbull	2008	Germany	0	0	0
60	Potenzial	2012	Germany	0	0	1
61	Preciosa	2009	Czech Republic	0	0	0
62	Princeps	2012	Germany	0	0	1
63	Raduza	2006	Czech Republic	0	0	0
64	Rapsodia	2003	UK	1	0	1
65	Rheia	2001	Czech Republic	0	0	1
66	RW Nadal	2010	Czech Republic	0	0	1
67	Sailor	2011	Germany	0	0	0
68	Sakura	2007	Czech Republic	0	0	0
69	Salutos	2009	Germany	0	0	1
70	Secese	2009	Czech Republic	0	0	0
71	Seladon	2009	Czech Republic	0	0	0
72	Simila	2006	Czech Republic	0	0	0
73	Sulamit	2000	Czech Republic	0	0	0
74	Sultan	2008	Czech Republic	0	0	0
75	Svitava	2001	Czech Republic	0	0	0
76	Tiguan	2012	France	1	0	0
77	Tobak	2013	Germany	0	0	0
78	Trend	2002	Germany	0	0	0
79	Turandot	2012	Czech Republic	0	0	0
80	Vanesa	2013	Bulgaria	1	0	1
81	Windsor	2001	Germany	1	0	0
82	Zeppelin	2013	Germany	0	0	0
83	Alana	1997	Czech Republic	0	0	0
84	Alka	1995	Czechoslovakia	0	0	0
85	Apache	1999	France	0	0	1
86	Asta	1994	Czechoslovakia	0	0	0
87	Athlet	1996	Germany	0	0	0
88	Blava	1992	Slovakia	0	0	0
89	Boka	1995	Czech Republic	0	1	0
90	Brea	1996	Czech Republic	0	0	0
91	Bruneta	1996	Czech Republic	0	0	0
92	GK Bekes	2005	Hungary-Szeged	0	0	-
93	GK Kalasz	1996	Hungary-Szeged	0	0	-
94	GK Csillag	2005	Hungary-Szeged	0	0	-
95	GK Petur	1999	Hungary-Szeged	0	0	-
96	GK Rozi	2010	Hungary-Szeged	0	0	-
97	MvSuba	2002	Hungary-Martonvásár	0	0	-
98	Mv Magdalena	1996	Hungary-Martonvásár	1	0	-

99	MvCsaradas	1999	Hungary-Martonvásár	1	0	-
100	MvKolo	2006	Hungary-Martonvásár	0	0	-
101	MvMarsall	2001	Hungary-Martonvásár	1	0	-
	Total			13	1	28

Presence/absence of 1BL.1RS (Lr26-Yr9-Sr31), Lr34-Yr18 and Lr37-Yr17-Sr38 is indicated by 1/0, respectively. - : not tested

1BL.1RS wheat-rye translocation (Lr26-Sr31-Yr9) was proved to be present in 13 (12.8%) of all cultivars tested in this study (Table 4). Lr37-Yr17-Sr38 was the most frequent rust resistance gene identified in 28 (30.7%) from 91 cultivars registered in Czech Republic. Only in one case, in Czech cultivar called 'Boka', Lr34-Yr18 was found (Table 4). The distribution of these rust resistance genes alone and in combination across the tested European wheat cultivars is summarized in Table 5.

Table 5: The distribution of rust resistance genes across the tested European wheat cultivars

Origin	No of cultivars	1BL.1RS (Lr26-Yr9-Sr31)		Lr34-Yr18		Lr37-Yr17-Sr38		Lr26-Yr9-Sr31+Lr34-Yr18	
		No	(%)	No	(%)	No	(%)	No	(%)
Austria	3	0	0	0	0.0	0	0.0	0	0.0
Bulgaria	1	1	100	0	0.0	1	100	1	100
Czech Republic	31	1	3.2	1	3.2	6	19.3	1	3.2
Denmark	2	1	50	0	0.0	1	50	1	50
France	11	1	9.0	0	0.0	4	36.4	0	0.0
Germany	34	3	8.8	0	0.0	12	35.3	1	2.9
Hungary	10	3	30	0	0.0	-	-	-	-
Netherlands	4	1	25	0	0.0	2	50	0	0.0
Slovakia	1	0	0.0	0	0.0	0	0.0	0	0.0
United Kingdom	4	2	50	0	0.0	2	50	1	25
Total	101	13	12.8	1	0.9	28	30.7	5	5.4

Among tested Hungarian cultivars, 1BL.1RS (Lr26-Yr9-Sr31) was shown in 3 (30%) cultivars. Only 3 (8.8%) cultivars of Germany carried 1BL.1RS (Lr26-Yr9-Sr31) from 34 cultivars. Lr37-Yr17-Sr38 was found in 12 (35.3%) German cultivars followed by Czech Republic 6 (19.3%). Interestingly, the only one Bulgarian cultivar 'Vanesa' contained both Lr26-Yr9-Sr31 and Lr37-Yr17-Sr38 rust resistance genes (Table 5). Of all tested cultivars, five 'Brilliant' (Germany), 'Matchball' (Czech Republic),'Orlando' (Denmark), 'Rapsodia' (United Kingdom), 'Vanesa' (Bulgaria) were proved to have both 1BL.1RS (Lr26-Yr9-Sr31) and Lr37-Yr17-Sr38 (Table 5).

As the occurrence of Lr37-Yr17-Sr38 was the highest in tested cultivars, it was possible to study its spread through the time. The number and frequency in the three periods displays an increasing

tendency. Lr37-Yr17-Sr38 was detected only in one cultivar originated between1992-1999, while it was found abundantly in the cultivars originated after 2000 (Table 6).

Table 6: Frequency of Lr37-Yr17-Sr38 genes in wheat cultivars originated from European countries between 1992-2013

Year of registration	Number of cultivars investigated		Frequency of Lr37-Yr17-Sr38	
	Number	(%)	Number	(%)
1992-1999	9	9.8	1	11.1
2000-2006	29	31.8	6	20.7
2007-2013	53	58.2	21	39.7

Discussion

Rust resistance wheat cultivars in Czech Republic and Hungary have contributed in reducing the probability of rust epidemics. The result of this study showed that Lr26-Yr9-Sr31 (12.87%) and Lr37-Yr17-Sr38 (30.7%) rust resistance genes were distributed in several European wheat cultivars. Lr37-Yr17-Sr38 is the most frequent gene among the postulated gene in registered cultivars. As it was reported by Hanzalova et al., (2013) among 19 Czech Republic wheat cultivars tested with molecular markers 10 cultivars possessed Lr37-Yr17-Sr38. Currently, the most frequent adult plant resistance gene in Czech Republic wheat cultivars is Lr37-Yr17-Sr38 (Chrpova et al., 2012). Durable rust resistance conferred by minor genes is also considered very important now a days. Lr34-Yr18 and Lr37-Yr17-Sr38 are among the most important genes which confer resistance to more than one of the wheat rusts. These adult plant resistance genes provide resistance to wider pathogen races than seedling resistance and conceived to be durable resistance.

Conclusion and recommendations: The result of this study indicates the potential use of molecular markers to screen rust resistance genes in abundant numbers of wheat cultivars for several resistance genes with reduced time and labour requirement. Marker assisted selection is environmentally independent method particularly useful for APR (adult plant resistance) genes that are hard to identify phenotypically at the seedling stage. However the use of both conventional inoculation and MAS is the most preferred and most reliable. It is suggested that cultivars that are tested in this paper using molecular markers should be supported by field artificial inoculation evaluations to the validation of DNA markers. The information presented here will be quite helpful in utilization of the identified resistance gene sources for further enhancement of genetic resistance and more over to design crossing programs for deploying

durable resistance genes in the respective zones and at the breeding centers. The methodology used in this study and MAS can be also applied in Ethiopia to explore rust genetic resistance in wild, cultivated and current cultivars.

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